Behavioral state modulates olfactory perception and behavioral response: serotonergic and peptidergic signaling interact to modulate aversive olfactory behaviors in Caenorhabditis elegans

Gareth P Harris
The University of Toledo

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by
Gareth P. Harris

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Molecular and Cellular Biology

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Professor Richard W. Komuniecki, Advisor

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Dr Patricia Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2010
An Abstract of

Behavioral State Modulates Olfactory Perception and Behavioral Response: Serotonergic and Peptidergic Signaling Interact to Modulate Aversive Olfactory Behaviors In Caenorhabditis elegans

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The University of Toledo
August 2010

Serotonin (5-HT) regulates diverse central and peripheral responses, including aggression, appetite, circadian rhythms, mood, and perception and a variety of drugs modulate serotonergic signaling in the treatment of depression, migraine, and schizophrenia. However, due to the complexity of the mammalian nervous system, the mechanisms underlying the serotonergic modulation are still only poorly understood. In the model nematode, Caenorhabditis elegans, behavioral state or “mood” is dependent on food availability and is translated by both monoaminergic and peptidergic signaling in the fine-tuning of most behaviors. We have used C. elegans to examine the interaction of monoamines and peptides in the modulation of aversive behaviors mediated by a pair of polymodal, nociceptive ASH sensory neurons. In the present study I have identified three different 5-HT receptors, SER-1, SER-5 and MOD-1, that operate at different levels
in the ASH mediated circuit are each essential for the serotonergic stimulation of aversive responses mediated by the ASH sensory neurons. In addition, using a combination of classical genetics and neuron-specific RNAi knockdown we have defined the G-protein signaling pathways involved in ASH sensitization and identified neuropeptides encoded by \textit{nlp}-3 to be essential for 5-HT dependent increases in octanol avoidance. Finally, I have begun to dissect the food signal in order to understand how food translates nutritional status through serotonergic signaling originating from multiple classes of serotonergic neurons and through neuropeptidergic signaling that are both required for changes in behavioral plasticity. Given that many of the \textit{C. elegans} peptide and monoamine receptors have clear orthologues in humans, these studies have the potential to provide novel insights into the peptidergic/aminergic modulation of human behaviors, such as appetite, mood and circadian rhythm.
My thesis is dedicated to my family, who have supported and encouraged me greatly through all my high school, college and university years.

I thank them dearly.
ACKNOWLEDGEMENTS

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<tr>
<td>AADC</td>
<td>Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>ACY-1</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AIA/Z</td>
<td>Amphidial interneuron</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor</td>
</tr>
<tr>
<td>ASH</td>
<td>Amphidial sensory neurons</td>
</tr>
<tr>
<td>ADF</td>
<td>Amphidial sensory neurons</td>
</tr>
<tr>
<td>AWB</td>
<td>Amphidial sensory neurons</td>
</tr>
<tr>
<td>BSR</td>
<td>Basal slowing response</td>
</tr>
<tr>
<td>BAs</td>
<td>Biogenic amines</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>daf</td>
<td>Dysregulation of dauer arrest mutants</td>
</tr>
<tr>
<td>DCV</td>
<td>Dense core vesicle</td>
</tr>
<tr>
<td>DGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DID</td>
<td>1, 1'-dioctadecyl-3, 3, 3'-tetramethylindocarbocyanine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
</tbody>
</table>
EAT-4  BNPI vesicular glutamate transporter
EAT-16 Regulator of G-protein subunit (RGS)
ESR Enhanced slowing response
EGL-8 Phospholipase C
EGL-3 Pre-protein convertase
EGL-21 Carboxypeptidase E
EGL-30 Go_q subunit
GFP Green fluorescent protein
GluC Glutamate-gated chloride channel
GOA-1 Go_o subunit
GPCR G-protein coupled receptor
GSA-1 Go_s subunit
HSN Hermaphrodite specific neurons
IBS Irritable bowel syndrome
INS Insulin like
KIN-1 PKA catalytic subunit
KIN-2 PKA regulatory subunit
MAO Monoamine oxidase
MGC Macro-Glomerular Complex
MOD-5 Serotonin reuptake transporter
MOD Modulation of locomotion defective
NGM Nematode growth media
NMDA N-methyl-D-aspartate
<table>
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<th>Full Name</th>
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<td>NPF</td>
<td>Neuropeptide F</td>
</tr>
<tr>
<td>NPR</td>
<td>Neuropeptide receptor</td>
</tr>
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<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NLP</td>
<td>Neuropeptide-like</td>
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<tr>
<td>NSMs</td>
<td>Neurosecretory motorneurons</td>
</tr>
<tr>
<td>OA</td>
<td>Octopamine</td>
</tr>
<tr>
<td>OAR</td>
<td>Octopamine receptor</td>
</tr>
<tr>
<td>OCTR-1</td>
<td>Octopamine receptor 1</td>
</tr>
<tr>
<td>ODR</td>
<td>Odorant defective</td>
</tr>
<tr>
<td>OSM-9</td>
<td>TRP transient receptor potential channel</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PDE-4</td>
<td>5’- cAMP phosphodiesterase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RIA</td>
<td>Ring interneuron A</td>
</tr>
<tr>
<td>RIH</td>
<td>Ring interneuron H</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G-protein signaling</td>
</tr>
<tr>
<td>RMDs</td>
<td>Ring motor neurons D class</td>
</tr>
<tr>
<td>rrf</td>
<td>RNA-dependent RNA polymerase family</td>
</tr>
<tr>
<td>SER</td>
<td>Serotonin receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>SV</td>
<td>synaptic vesicle</td>
</tr>
<tr>
<td>ttx</td>
<td>Thermotaxis defective</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TPH-1</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transformer growth factor Beta</td>
</tr>
<tr>
<td>UNC-13</td>
<td>DAG-binding protein</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5-HP</td>
<td>5-Hydroxytryptophan</td>
</tr>
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</table>
LIST OF SYMBOLS

$\alpha$ - Alpha

$\beta$ - beta
1.0 CHAPTER I

OVERVIEW/SIGNIFICANCE

In mammals, serotonin (5-HT) regulates diverse central and peripheral responses, including, aggression, appetite, circadian rhythm, mood, perception, and pain, as well as participating in homeostatic processes, such as, gastrointestinal peristalsis, blood coagulation, and blood pressure. A variety of drugs are available that modulate serotonergic signaling in the treatment of depression, migraine, hyperanalgesia, schizophrenia, irritable bowel syndrome, and cardiac arrhythmia; alcoholism, anorexia, Parkinson’s disease, ulcerative colitis and schizophrenia (Bearcroft et al., 1998; Lucki, 1998; Mockus and Vrana, 1998; Barnes and Sharp, 1999; Spiller et al., 2008; Sibille and Hen, 2001; Heisler et al., 2007; Heal et al., 2008). However, the mechanisms underlying the serotonergic modulation of these behaviors are still largely unknown, and our understanding of the role of serotonergic signaling on synaptic plasticity is still in its infancy (Lucki, 1998; Lesch, 2001; Durham, 1998, Heisler et al., 2006; Heisler et al., 2007). In addition, little is known about how serotonergic and peptidergic signaling interacts to modulate synaptic transmission. The complexity of the mammalian nervous system makes it extremely difficult, if not impossible, to dissect the mechanisms underlying serotonergic signaling.
The nematode *Caenorhabditis elegans* is used as a model to examine aspects of neuronal signaling, with its sequenced genome and advanced genetics providing experimental advantages not present in other models. The *C. elegans* nervous system contains only 302 neurons, with synaptic connectivities and neurotransmitters previously mapped and characterized (White et al., 1986). Most importantly, many aspects of monoaminergic modulation have already been defined and most, if not all of the key monoamine receptors have been identified, characterized and at least partially localized (Rex and Komuniecki, 2004; Hamdan et al., 2000; Olde and McCoombie, 1999; Hobson et al., 2003; Wragg et al., 2007). Finally, a variety of sensory-mediated locomotory assays have been developed that are sensitive to nutritional status, behavioral state and the addition of exogenous monoamines.

### 1.1 5-HT regulates a number of processes in mammals and invertebrates

5-HT is implicated in aggression, anorexia, appetite, arousal, circadian rhythm, memory, mood, pain and sleep, as well as integrating complex brain functions such as cognition, sensory processing, learning, motor activity and olfactory perception (Wilkinson and Dourish, 1991; Buhot et al., 2000; Milan, 2002; Heisler et al., 2002; Glass et al., 2003; Gaspar et al., 2003; Garfield and Heisler, 2009).

In invertebrates, 5-HT is implicated in an array of processes. For example, in insects, 5-HT modulates the visual system, circadian rhythms, reproduction, feeding, heart rate, olfactory processing, locomotion and sleep (Kamyshev et al., 1983; Novak, 1995; Yuan et al., 2005; Dacks et al., 2009). The sensitivity of olfactory sensory cells in invertebrates is also regulated by 5-HT (Paztor, 1989). For example, in the marine snail,
Aplysia californica, 5-HT increases the sensitivity of olfactory neurons (Goldsmith and Abrams, 1991). 5-HT also increases the sensitivity of mechanosensory neurons and the responsiveness of olfactory neurons to external stimuli, such as pheromones in Aplysia californica and Bombyx mori (Braha et al., 1990; Byrne and Kandel 1996; Gatellier et al., 2004). Both the excitability of MGC projections and responses to pheromonal stimuli in the male Sphinx moth are enhanced by serotonergic signaling, as well as olfactory glomeruli development in Manduca sexta and modulation of neurons of the olfactory centre (Siegelbaum et al., 1982; Mercer et al., 1995; Kloppenburg et al., 1999). In Drosophila melanogaster, 5-HT also enhances sensitivity of the antennal lobe output projection neurons in an odor-specific manner (Dacks et al., 2009). However, in all cases, the mechanisms underlying this serotonergic signaling, i.e, the 5-HT receptors and downstream effectors that modulate these processes, are still only poorly defined.

1.2 5-HT modulates multiple behaviors in C. elegans

In C. elegans, 5-HT defines nutritional state and food availability in combination with other monoamines and peptides. 5-HT is secreted from nine hermaphrodite serotonergic neurons that were first identified by formaldehyde-induced fluorescence and antibodies against 5-HT, the ADFs (amphidial sensory neurons), NSMs (pharyngeal neurosecretory motor neuron), HSNs (hermaphrodite specific neurons), AIMs and RIH (Horvitz et al., 1982; Sawin et al., 2000; Sze et al., 2000, Hare and Loer, 2004; Zheng et al., 2005). 5-HT is synthesized from L tryptophan (Figure 1). L-tryptophan is hydroxylated by tryptophan hydroxylase that is encoded by tph-1 in C. elegans to make 5-hydroxy-L-tryptophan (5-HTP), which is in turn decarboxylated by 5-
hydroxytryptophan decarboxylase encoded by bas-1 to produce serotonin (5-HT) (Loer and Kenyon, 1993). TPH-1 is the key enzyme involved in biosynthesis of 5-HT and is expressed in some, but not all of the C. elegans serotonergic neurons (Sze et al., 2000). Presumably the other serotonergic neurons acquire their 5-HT by transporter-dependent uptake (MOD-5) after secretion from the 5-HT synthesizing neurons. For example, tph-1 expression has been observed in the NSMs, ADFs, HSNs and occasionally the RIHs and AIMs, but the signals that modulate serotonergic release are still unknown (Sawin et al., 2000; Sze et al., 2000; Loer and Rivard, 2007). The NSMs mediate the enhanced slowing of starved animals in response to food (potentiation of the enhanced slowing response by fluoxetine requires serotonin from the NSMs and the inhibition of MOD-5 dependent reuptake), dispersal off food, male tail curling and turning during male mating (Avery et al., 1993; Avery and Thomas, 1997; Sawin et al., 2000; Lipton et al., 2004; Wakabayashi et al., 2004). However, although 5-HT dramatically stimulates pharyngeal pumping, ablation of the NSMs has no obvious effect on pumping or feeding, suggesting that the primary function of the NSMs remains to be determined. Alternatively, removal of all outputs from the NSM, i.e. 5-HT/peptides and glutamate masks the individual effects of these signaling molecules on pharyngeal pumping (Avery and Horvitz, 1989). The NSMs are thought to sense food, generating a widespread humoral effect similar to the effects produced by epinephrine when secreted from the adrenal medulla (Kemppaires and Behrend, 1997). The ADFs have been implicated in 5-HT dependent increases in aversive learning to pathogenic bacteria, dispersal off food, inhibition of reversal behavior, increasing forward movement, chemotaxis, thermotaxis, entry into dauer and foraging behavior. The ADFs also appear to act as a convergence point for the
regulation of hypoxia avoidance (Horvitz et al., 1982; Bargmann and Horvitz, 1991; Jansen et al., 1999; Wakabayashi et al., 2004; Wakabayashi et al., 2005; Zhang et al., 2005; Chang et al., 2006; Hukema et al., 2008; Dernovici et al., 2006; Zubenco et al., 2008). Recent work suggests that serotonergic signaling provides both excitatory and inhibitory input to neural circuit function, and more interestingly, that 5-HT originating from the NSMs and ADFs may act antagonistically on nutrition dependent behavior, with food stimulating NSM dependent 5-HT signaling and stress and starvation stimulating ADF 5-HT signaling (Chang et al., 2006; Harris and Komuniecki, unpublished).

Interestingly, tph-1 animals are defective in serotonin signaling, but still remain viable on NGM plates, suggesting that other signaling pathways may play redundant roles (Sze et al., 2000; Hapiak et al., 2009).
**Fig. 1: Serotonin biosynthesis pathway.** Diagram represents the biosynthesis of serotonin (5-HT) in mammals. There are two key enzymes identified that operate in the synthesis of 5-HT, 5-tryptophan hydroxylase and hydroxytryptophan decarboxylase, encoded by *tph-1/tph-2* and *aadc*, respectively. In *Caenorhabditis elegans*, there are two homologues, TPH-1 and BAS-1 required for serotonin synthesis, that are encoded by *tph-1* and *bas-1* that encode tryptophan hydroxylase and serotonin-dopamine-synthetic aromatic amino acid decarboxylase (AAADC), respectively.

5-HT levels in *C. elegans* are predicted to increase when a wild-type animal encounters bacteria and serves as the “food is at hand” signal, modulating in combination with peptides and other monoamines such as dopamine, most food-related behaviors (Table.1). 5-HT 1) stimulates an enhanced slowing response after a starved wild-type animal encounters food (Horvitz et al., 1982), 2) stimulates egg-laying by initiating the contraction of vulval muscle (Horvitz et al., 1983; Waggoner et al., 1998; Carnell et al., 2005) and 3) increases the rate of pharyngeal pumping by stimulation of the MC and M3 pharyngeal motor neurons, resulting in rapid cycles of contraction and relaxation that increases the overall rate of pumping (Niacaris and Avery, 2003; Carnell et al., 2005; Dempsey et al., 2005). 5-HT also plays a role in other more complex behaviors, such as aversive learning to pathogenic bacteria, chemoattraction or olfactory adaptation to benzaldehyde, olfactory imprinting, NaCl chemotaxis and plasticity, tail curling and turning during male mating (Loer and Kenyon, 1993; Colbert and Bargmann, 1995; Duerr et al., 1999; Nuttley et al., 2002; Remy and Hobert, 2005; Zhang et al., 2005; Hukema et al., 2008; Tsui and Vanderkooy, 2008). 5-HT is also implicated in the development of touch avoidance circuits, affects neuronal migration during development,
and modulates defecation rate and longevity (Lui and Thomas, 1994; Segalat et al., 1995; Weinshenker et al., 1995; Dal Santo, 1999; Kindt et al., 2002; Zhang et al., 2005; Murakami and Murakami, 2007). Locomotory behaviors are also controlled by 5-HT; for example, 5-HT appears to stimulate forward locomotion, based on decreased duration of forward movement exhibited by mutants in serotonin biosynthesis enzymes, tph-1 and bas-1 and examination of animals containing ablated NSMs/ADFs and surprisingly HSN serotonergic neurons (Wakabayashi et al., 2004). Food also modulates reversal frequency through serotonergic signaling based on defective reversal frequency observed in serotonin synthesis and 5-HT receptor null mutants (Tsalik and Hobert, 2003; Dernovici et al., 2007; Harris et al., 2009). 5-HT also stimulates foraging behavior and the head withdrawal reflex (Elkes and Kaplan, 1990). In contrast, at higher concentrations, ectopically applied 5-HT (>15 mM) dramatically decreases locomotory rate and can cause a flaccid paralysis. Exogenous 5-HT inhibits synaptic transmission at neuromuscular junctions by at least indirectly inhibiting acetylcholine release (Horvitz et al., 1982; Segalat et al., 1995). Although whether 5-HT functions directly in the motorneurons or upstream in interneurons remains to be determined. 5-HT deficient mutants also have excess fat accumulation, suggesting that 5-HT interacts with the TGF-B and/or insulin signaling (Avery and Horvitz, 1990).
<table>
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<td>McDonald et al., 2007; Wakabayashi et al., 2005, Gray et al., 2005</td>
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<td>Croll et al., 1975; Gray et al., 2005, Tsalik and Hobert, 2003; Bendena et al., 2008; Wakabayashi et al., 2005?</td>
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<td>DA, Neuropeptides, 5-HT</td>
<td>Tsalik and Hobert, 2003, Grey et al., 2005; Bendena et al., 2008; Dernovici et al., 2007</td>
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**Table 1. Food modulates a variety of behaviors in C. elegans.** Food dependent effects are mediated through at least aminergic signaling i.e. 5-HT or DA, and/or peptidergic signaling. Table indicates behaviors modulated by food, amines that are implicated in food dependent modulation and indication of any role for neuropeptides in each behavior. References sited have previously examined the above behaviors and determined a possible role for aminergic or peptidergic signaling in these behaviors.
1.3 5-HT signals through multiple 5-HT receptors in C. elegans

In C. elegans, 5-HT signals through multiple 5-HT receptors to modulate simple and complex behaviors. At least five C. elegans 5-HT receptors have been identified, four metabotropic G protein coupled 5-HT receptors, SER-1 (5-HT$_2$-like mammalian receptor), SER-4 (5-HT$_1$-like mammalian receptor) and SER-7 (5-HT$_7$-like mammalian like receptor) that appear to couple to Gα$_q$, Gα$_o$ and Gα$_s$ respectively, and MOD-1 a 5-HT gated chloride channel (Olde and McCombie, 1997; Hamdan et al., 1999; Ranganathan et al., 2000; Hobson et al., 2003). The fifth 5-HT receptor, SER-5, (5-HT$_6$-like) is still to be completely characterized (Hapiak et al., 2009; Harris et al., 2009; Harris et al., 2010). 5-HT dependent modulation is complex and often requires multiple receptors. More importantly, 5-HT modulation provides both excitatory and inhibitory input into most processes, regardless of the phenotype observed by the addition of exogenous 5-HT. For example, both SER-1 and SER-7 are required in vulval muscle for the 5-HT stimulation of egg-laying (Hobson et al., 2003; Xiao et al., 2006). In contrast, the addition of 5-HT to ser-1;ser-7 null animals actually inhibits egg-laying on bacteria through an inhibitory pathway requiring SER-4 and MOD-1. Finally, the addition of 5-HT to ser-1;ser-7;mod-1;ser-4 animals again stimulates egg-laying, through SER-5 in vulval muscle, highlighting the layers of serotonergic signaling modulating egg-laying. Similarly, both excitatory and inhibitory 5-HT receptors have been identified in the 5-HT stimulation of pharyngeal pumping (Hobson et al., 2003; Niacaris and Avery, 2003).

For example, SER-1 is essential for egg-laying, enhanced slowing response, longevity, male mating, pharyngeal pumping and reversal frequency on and off food (Hobson et al., 2003; Carnell et al., 2005; Dempsey et al., 2005; Dernovici et al., 2007;
Harris et al., 2009). SER-4 is implicated in 5-HT dependent inhibition of locomotion (enhanced slowing response), male tail curling, foraging, inhibition of egg-laying and neurotransmitter release at the NMJ has been characterized (Horvitz et al., 1982; Dempsey et al., 2005; Carret-Pierrat et al., 2006; Hapiak et al., 2009). SER-7 is required for stimulation of egg-laying or pharyngeal pumping by serotonin, for regular pumping in response to bacteria, and probably for 5-HT to activate MC neurons (Hobson et al., 2003). MOD-1 is important in the enhanced slowing response, inhibition of egg-laying, inhibition of reversals off food, stimulation of reversals and aversive learning responses to various forms of pathogenic bacteria and chemoattractive behavior to benzaldehyde in aged animals (Ranganathan et al., 2004; Carnell et al., 2005; Zhang et al., 2005; Dernovici et al., 2007; Harris et al., 2009; Tsui and Van der Kooy, 2008). SER-5 may play a subtle role in the stimulation of egg-laying in the vulval muscle (Hapiak et al., 2009). Overall, 5-HT modulates multiple behaviors through one or more 5-HT receptors operating neuronally or/and on body wall, vulval or pharyngeal muscle. Little is known about the signaling downstream of these receptors. However, despite the involvement of 5-HT in these behaviors, the functional localization and downstream signaling of the five previously characterized C. elegans 5-HT receptors (SER-1, 4, 5, 7 and MOD-1) are still largely unknown. Interestingly, many of these 5-HT receptors are expressed on neurons that are not directly innervated by serotonergic neurons, suggesting that a significant portion of serotonergic signaling is probably humoral and extra-synaptic.

1.4 Neuropeptides modulate a number of processes in mammals and invertebrates
In mammals, neuropeptides have been implicated in an array of processes, that include appetite, cognition, energy balance, fat digestion, memory and learning, pain and perception, vomiting reflex, vasodilation or vasoconstriction of blood vessels and water retention in the kidneys all involve peptidergic signaling (Hornby, 2001; Cone, 2005; Colmers and Bahh, 2003; Seybold et al., 2003). In addition, neuropeptides have been linked to a number of disorders, including Alzheimer’s, ADHD, anorexia, anxiety, dementia, obesity, obsessive-compulsive disorder and schizophrenia (Altemus et al., 1992; Wahlesredt et al., 1993; Zhou et al., 2008). In mammals, the mechanisms behind the peptidergic modulation of neuronal plasticity are still poorly understood, but they appear to modulate both pre-synaptic release and post-synaptic sensitivity. For example, somatostatin, substance P, calcitonin gene related peptide (CGRP), vaso-active intestinal peptide (VIP) and neuropeptide Y (NPY) are found in sympathetic neurons that are primary referred to as cholinergic neurons. Substance P modulates NMDA receptor mediated responses by modulating long-term protein synthesis in the mouse spinal cord (Hornfeldt et al., 1994). Similarly, NPY inhibits transmitter release from both cholinergic and noradrenergic nerves in the heart (Kilborn et al., 1985; Tay and Wong, 1992). Insulin, CGRP and substance P signaling all appear to modulate glutamatergic synapses through the regulation of the abundance of synaptic/surface AMPARs (Man et al., 2000; Esteban et al., 2003). Neuropeptides also modulate postsynaptic sensitivity and in parasympathetic submandibular ganglion neurons modulate voltage-gated Ca\(^{2+}\) channels (Endoh, 2004).

Neuropeptides also are important modulators in invertebrates. For example, CRH, CCK, NPY, neuropeptide F, insulin, glucagons, gastrin, vasopressin, endorphins and
enkaphalins all have apparent homologues in invertebrates and in insects some of these neuropeptides appear to have actions similar to those of the corresponding vertebrate peptides (Strand, 1999). Neuropeptides have been extensively studied in the parasitic nematode, *Ascaris suum* and can have both excitatory and inhibitory effects on locomotion, pharyngeal pumping and ovijector activity in *A. suum* (Cowden et al., 1989; Brownlee et al., 1997). Overall, neuropeptides play a key role in the modulation of multiple behaviors in vertebrates and invertebrates, but in almost all cases their molecular mode of action is still unknown.

### 1.5 Neuropeptides in *C. elegans*

In *C. elegans*, neuropeptides have been classed into three specific families, insulin-like peptides, FMRF-amides (Phe-Met-Arg-Phe-NH2) or FaRPs and neuropeptide-like peptides and have been implicated in chemosensation, mechanosensation, osmotic balance, lifespan, egg-laying, ethanol response, locomotion, pharyngeal pumping, male turning and social behavior, fat metabolism, feeding and adaptive changes in sensory responses (Kaplan and Horvitz, 1993; Mori and Ohshima, 1995; Nelson et al., 1998; de Bono and Bargmann, 1998; Kubiak et al., 2008; Rogers et al., 2003). The *C. elegans* genome contains 113 putative neuropeptide genes that encode at least 250 peptides, 20 *FaRPs* (FMRFamide-like), 47 *nlp*s (neuropeptide like) and 42 *ins* (insulin-like) peptides (Li et al., 1999; Duret et al., 1998; Gregoire et al., 1998; Kawano et al., 2000; Nathoo et al., 2001; Husson et al., 2007). However, receptors for only 9 of these peptides have been identified to date and even these associations are based on heterologous expression, often at unphysiologically high concentrations of peptide (de Bono and Bargmann, 1998;
Neuropeptides are processed by type 2 pre-protein convertases, and carboxypeptidase E-like enzymes. Both enzymes process specific neuropeptide precursors and are broadly expressed in the *C. elegans* nervous system (Kass et al., 2001; Jacob and Kaplan, 2003). Four pre-protein convertases (KPC1, EGL-3, AEX-5 and BLI-4) and 3 carboxypeptidases (including EGL-21) have been identified in *C. elegans* (Thacker and Rose, 2000; Husson et al., 2006). Animals defective in peptide processing exhibit distinctive behavioral phenotypes, i.e. EGL-3 has been implicated in egg-laying, locomotion and mechanosensation. In addition, *egl-3* mutations rescue effective nose touch responses observed in *glr-1* mutants, suggesting that peptides inhibit glutamate signaling either by modulating postsynaptic neurotransmitter receptors or, alternatively and more probably, decreasing neurotransmitter release from presynaptic neurons (Kass et al., 2001; Mellem et al., 2002).

EGL-21 appears to be important in regulating acetylcholine transmission at neuromuscular junctions, as *egl-21* null mutants exhibit decreased sensitivity to the nicotinic acetylcholine receptor agonist, levamisole. In addition, *egl-21* animals exhibit phenotypes associated with locomotion, mechanosensation, defecation, reversal frequency and omega turns (Jacob and Kaplan, 2003; Harris and Komuniecki, unpublished). *egl-21* and *egl-3* null animals also exhibit defective male turning frequency during mating, abnormal locomotion and exhibit defects in the ability to interpret and integrate starvation signals (Liu et al., 2007). For example, *flp-1* is necessary for the regulation of locomotion, egg-laying, food recognition, fat metabolism,
osmolarity and nose touch on food (Nelson et al., 1998; Waggoner et al., 2000). Peptides encoded by \textit{flp}-18 modulate chemosensory responses to near threshold odors through their predicted neuropeptides receptors, \textit{npr}-4/5 (Cohen et al., 2009). FMRFamide peptides expressed in pharyngeal neurons have also been implicated in the modulation of pharyngeal pumping. For example, peptides encoded by \textit{flp}-3, 8 and 13 inhibit pharyngeal pumping and those encoded by \textit{flp}-17 stimulate pharyngeal pumping (Rogers et al., 2003; Papaioannou et al., 2008). In addition, peptides encoded by \textit{flp}-18/\textit{flp}-21 are also involved in the suppression of social feeding behavior (de Bono and Bargmann, 1998; Rogers, 2003), and those by \textit{flp}-21 are required for the enhanced slowing response (Jacob and Kaplan, 2003). Peptides encoded by \textit{npr}-9 (galanin/allatostatin like receptor) appear to promote roaming behavior on food (Bendena et al., 2008), and by \textit{nlp}-1 and \textit{npr}-11 for AWC-mediated chemosensory behaviors (Chalasani et al., 2010). \textit{npr}-1 also mediates aspects of food-dependent behaviors. For example, \textit{npr}-1 inhibits aggregation on high levels of bacteria, inhibits hyperoxia avoidance, inhibits social behavior, promotes carbon dioxide avoidance, regulates foraging and dispersal strategies and modulates adaptation to ethanol (de Bono and Maricq, 2001; Cheung et al., 2004; 2005; Gloria-Soria and Azevedo, 2008; Bretcher et al., 2008; Macosko et al., 2009).

Putative expression patterns have been determined for most \textit{flps}, \textit{nlp}s and \textit{ins} genes, using \textit{gfp} transgenes containing minimal putative promoters. For example, \textit{nlp}-3, \textit{nlp}-15, \textit{flp}-21 and \textit{ins}-1 are expressed in the ASH neurons and multiple \textit{flps} (\textit{flp}-1, 5, 7, 21, 18, 21, 22, and \textit{ins}-1) are expressed in postsynaptic partners of the ASH (Li et al., 1999; Nathoo et al., 2003; Husson et al., 2005). These peptides may cell autonomously regulate 1) neuronal excitability, 2) the release of classical transmitters, such as glutamate, and/or
peptides or 3) the activity state of the postsynaptic cells either by acting synaptically as neurotransmitters on neurons in the ASH-mediated circuit and/or by acting as neurohormones and diffusing throughout the pseudocoelomic fluid to modulate cells distant from their site of release (Malcangio and Bowery, 1999).

Despite a limited knowledge of neuropeptides and neuropeptide receptors required for select behaviors, very little is known on the specific sites of peptide release from neurons, the mechanisms modulating peptide release, or peptide receptor signaling pathways. In addition, it is unclear if the neuropeptides required for nutritional state-dependent behaviors are tonically released or are differentially regulated by the availability of food. Certainly all of these possibilities and more are operating, but unfortunately almost nothing is known about the specifics of these processes.

1.6 C. elegans is a good model for studying olfaction

*C. elegans* has proven to be particularly useful as a model to investigate the complex behavioral repertoires modulating olfaction (Wood et al., 1988; Sengupta et al., 1996). *C. elegans* hermaphrodites contain only 302 neurons and its synaptic connectivities and neurotransmitters have been well documented. *C. elegans* contains 12 bilaterally symmetric pairs of sensory neurons in the head that act primarily as chemosensors (ADFs, ADLs, AFDs, ASKs, ASEs, ASGs, ASIs, ASJs, ASHs, AWAs, AWBs and AWCs). These neurons project along two anterior structures known as amphids. The tail contains two additional pairs of sensory neurons (PHAs and PHBs), that are involved in directional integration of sensory inputs (Ward et al., 1975; Ware et
al., 1975; Hilliard et al., 2005). *C. elegans* responds to a variety of stimuli through well-defined chemosensory, mechanosensory and thermosensory pathways (Ward et al., 1975). *C. elegans* detects water soluble attractants in the micromolar range, including Na\(^+\), K\(^+\), Cl\(^-\), OH\(^-\) and water-soluble cGMP and cAMP (Dubendorf et al., 1992; Bargmann et al., 1993). *C. elegans* senses organic attractants, such as thiazide, diacetyl, butanone, benzaldehyde and iso-amyl alcohol (Bargmann et al., 1993; Sengupta et al., 1996) and volatile repellents, including nonanone, octanol, quinine, SDS and mechanosensory stimuli, such as nose touch and osmotic repellents (Troemel et al., 1995; Sengupta et al., 1996; Hilliard et al., 2002). The ASH sensory neurons are polymodal and detect an array of noxious stimuli, including volatile repellents, salts, nose touch, and hyper-osmolarity (Bargmann et al., 1990; Kaplan and Horvitz, 1993; Hart et al., 1995; Troemel et al., 1995; Sambongi et al., 1999; Hilliard et al., 2002; Chao et al., 2004, Thiele et al., 2009). The mechanisms behind sensory transduction in individual sensory neurons and the neural circuits mediated by these sensory neurons have been extensively described and serve as a starting point to study the role of aminergic/peptidergic modulation proposed in the present studies (Tsalik and Hobert, 2003; Gray et al., 2005; Wakabayashi et al., 2004; Wragg et al., 2007; Chalasani et al., 2007, 2010; Harris et al., 2009, 2010).

1.7 5-HT modulates responses to chemosensory behaviors in *C. elegans*

The ASH sensory neurons are necessary and sufficient for responses to dilute octanol and these ASH-mediated aversive responses are stimulated by the presence of food or exogenous 5-HT (Chao et al., 2004). For example, laser ablation of the ASH
sensory neurons abolishes responses to dilute octanol (Chao et al., 2004). Aversive responses to 100% octanol in wild-type animals are independent of food or 5-HT and requires a more complex chemosensory pathway, including input from at least two additional pairs of sensory neurons, the AWBs and ADLs, that may provide inhibitory input into the ASH mediated circuit (Nathoo et al, 2001; Chao et al., 2004).

Food or 5-HT appears to stimulate the ASHs directly, as ASH calcium transients in response to nose touch can only be measured in the presence of 5-HT (Hilliard et al., 2005). However, based on a limited analysis of ser-x::gfp transcriptional and translational fusions, none of the currently identified 5-HT receptors, SER-1, SER-4, SER-7 and MOD-1, appear to be expressed in the chemosensory sensory neurons (Ranganathan et al., 2000; Hobson et al., 2003; Tsalik et al., 2003; Xiao et al., 2006; Dernovici et al., 2007). This result suggests that an uncharacterized 5-HT receptor may be responsible for the serotonergic stimulation of the ASH and ASH mediated behaviors (Hilliard et al., 2005).

ASH signaling and its potential monoaminergic modulation is poorly understood. 5-HT could act at a number of levels within the ASHs or its associated aversive circuit to increase aversive responses. 5-HT could increase the amount or sensitivity of the unidentified octanol receptor, modulate dendritic signaling or neuron excitability downstream from the odorant receptor, modulate the release of glutamate or peptides or both, alter neurotransmitter release in a synapse-specific fashion or modulate the desensitization of components of the signaling system, since recent data suggests that the off signal from the sensory neuron may be most important in controlling the responses of the postsynaptic neurons (Chao et al., 2004; Murphy et al., 2004; Hilliard et al., 2005;
Gray et al., 2005; Chalassani et al., 2007). A number of components in the ASHs have been identified that are essential for basal responses to octanol. These include the heterotrimeric G proteins, ODR-3 and GPA-3, the voltage-gated calcium channel, EGL-19 (Jospin et al., 2002), and the TRP channel subunits, OSM-9/OCR-2 (Bargmann et al., 1990; Tobin et al., 2002; Hilliard et al., 2005). The sensitivity of any of the above signaling components may be modulated by serotonergic signaling. In addition, GPA-11, a novel ASH/ADL specific Gα subunit, appears to be essential for 5-HT dependent increases in sensitivity to dilute octanol but not basal aversive responses (Chao et al., 2004, Lans and Janssen, 2006; Harris and Komuniecki, unpublished). However, the role of this novel Gα subunit in ASH signaling or serotonergic sensitization is unclear.

1.8 5-HT could also increase sensitivity to dilute octanol through the modulation of downstream interneurons.

The major postsynaptic partners of the ASHs are the AIA, AIB and RIA interneurons, the AVA and AVD backward command interneurons and the AVB forward command interneurons, although other, less studied connections are also apparent, including the AVEs, ADFs, RIMs, HSNs and RMGs. The AIAs and AIBs integrate signals directly from the sensory neurons to modulate the dynamics of locomotion, i.e., forward/backward movement or turns in response to changes in environmental conditions. Simply stated, these interneurons are key to the translation of nutritional status and food availability perceived by an array of sensory neurons into one of the three clearly defined locomotory states manifested on food (dwelling with frequent
pivots), immediately off food (area restricted search (ARS), long reversals) or during starvation (long dispersal or roaming) (Tsalk and Hobert, 2003; Gray et al., 2005;).

The ablation of many of the neurons in the ASH-mediated circuit alters reversal behavior. For example, AIB, AIZ or AVA ablated animals exhibit a hyporeversal phenotype (Chalfie, 1985; Tsalk and Hobert, 2003). Suggesting that these 3 pairs of neurons may stimulate spontaneous reversals. In contrast, the ablation of the AIYs, RIMs or AVBs produces a hyperreversal phenotype, suggesting that these neurons inhibit reversals (Chalfie, 1985; Gray et al., 2005). In addition, AVA ablation also inhibits long reversals (Wakabayashi et al., 2005).

Ablation of the AIAs or AIBs suggests that they are involved in reversal behavior (Gray et al., 2005). For example, the fine tuning of reversal frequency and turning behavior on and off food, distinct locomotory behaviors that are essential for area-restricted searches, and dispersals, as well as temperature sensing, social behavior and location of attractive stimuli such as salt (Hobert et al., 1997; Mori and Oshima, 1997; Tsalk and Hobert, 2003; Gray et al., 2005; Bendena et al., 2008). In addition, the RIM interneurons/motorneurons, major downstream partners of the AIBs, also play a pivotal role in the modulation of reversals, and subsequently the manipulation of the RIM interneuron/motorneurons, one of the AIBs main downstream partners, through laser ablation, expression of channel rhodopsin, or removal of tyraminergic signaling produces gross effects on locomotory behavior (Chalfie, 1985; White et al., 1986; Maricq et al., 1995; Tsalk and Hobert, 2003; Gray et al., 2005). The AVA, AVB, AVD and AVE command interneurons appear to function as a bistable switch to modulate forward and
backward locomotion (Chalfie, 1985; Zheng et al., 1999; Brockie et al., 2001; Tsalik and Hobert, 2003, Gray et al., 2004). The RIAs synapse onto RMDs/SMDs ring motor neurons that modulate the sensitivity of head muscle and are required for head oscillations and foraging behavior.

5-HT receptor expression has been tentatively localized by expression of ser-x::gfp transcriptional and translational fusions. For example, SER-1 is localized to the ventral cord motor neurons, ring motor neurons (RMDs), vulval muscle, as well as the ring interneurons, (RIAs, RICs) and PVC (Hamdan et al., 1999; Xiao et al., 2006; Dernovici et al., 2007). SER-4 is expressed in AIBs, RIBs, RIS, PVT, DVA, DVC neurons, possibly in the motor neurons, vulval muscle (as well as a few other potentially faintly fluorescing neurons in the nerve ring (Tsalik et al., 2003; Fox et al., 2005), MOD-1 in the AIA/AIB/AIZ/AIYs and PVC interneurons and possibly other neurons such as the ventral nerve cord and tail (Rangananthan et al., 2000; Dernovici et al., 2007) and SER-5 in an wide array of neurons in the head and tail, as well as vulval and body wall muscle. Interestingly, ser-5 expression was faintly observed in the AWB and ASH sensory neurons. However, it should be stressed that rescuing gfp translational fusions only approximate receptor expression and it is more than possible that low levels of receptor expression may be sufficient for rescue but not for GFP visualization. Indeed, DOP-1 appears to function in the ASHs to inhibit ASH-mediated aversive responses, based on neuron-specific rescue and RNAi, but dop-1::gfp expression could not be observed in the ASHs (Ezac et al., 2010).
1.9 Does 5-HT modulate glutamatergic transmission involved in the ASH-mediated responses to octanol?

Ten putative AMPA-like and 2 NMDA-like glutamate receptors are expressed in C. elegans, many in the command interneurons (AVA, AVB, AVD, AVE and PVC) that are essential for switches between forward and backward locomotion (Brockie et al., 2001; Brockie and Maricq, 2003). In addition, at least six genes encode subunits of inhibitory cys loop glutamate-gated chloride channels, *glc-1, glc-2, glc-3, glc-4, avr-14* and *avr-15* (Cully et al., 1994; Dent et al., 1997; Laughton et al., 1997a; Vassilatis et al., 1997; Dent et al., 2000; Brockie et al., 2001; Horoszok et al., 2001; Brockie and Maricq, 2003; Cook et al., 2006). Examination of mutants lacking genes encoding the glutamate vesicular transporters (*eat-4*) and glutamate receptors (*glr-1, glr-2, glr-3, glr-4, glr-5* and *glr-6*) has demonstrated clear roles for glutamatergic signaling in multiple locomotory behaviors (Brockie and Maricq, 2003). For example, GLR-1 is expressed in neural circuits that mediate avoidance behaviors and are required for glutamate-gated currents in the AVA and AVD interneurons (Walker et al., 2006). In addition, GLR-1 is involved in reversal (ARS), forward movement, foraging, and turning during male mating, turning behavior during chemotaxis, tap responses and responses to changes in osmolarity and nose touch (Hart et al., 1995; Maricq et al., 1995; Zheng et al., 1999; Mellem et al., 2002; Chao et al., 2004; Mano et al., 2007). NMR-1, an NMDA like glutamate receptor type is essential for responses to osmolarity and volatile anaesthetics (Mellem et al., 2002; Nagele et al., 2004). GluCs have also been implicated in a variety of locomotory behaviors such as reversal frequency, reversal duration, forward movement and turning behavior in response to chemotaxis (Cook et al., 2006; Chalasani et al., 2007, 2010;
Summers and Komuniecki, unpublished). Glutamatergic signaling in *C. elegans* is presumably synaptic, but *eat-4* is also expressed on some neurons such as the NSMs (neurosecretory motor neurons) that do not contain any evident neuron-neuron synapses, suggesting that glutamate may also be secreted in a neuroendocrine manner.

In the present study, I identified a subset of monoamine receptors that are each essential for 5-HT dependent increases in aversive responses to dilute octanol. These 5-HT receptors appear to operate at different levels within the ASH-mediated locomotory circuit to integrate sensory input with locomotory behavior, i.e, SER-5 in the ASH sensory neurons, MOD-1 in the AIB, and possibly AIY, interneurons that integrate signals directly from the ASH sensory neurons and SER-1 in the RIA interneurons, major upstream partners of the ring motor neurons that innervate head muscle. Based on the comprehensive wiring diagram of the *C. elegans* nervous system, we predict that SER-5 increases the responsiveness of the ASHs to dilute octanol, MOD-1 modulates interneuronal signaling to stimulate reversal and backward locomotion and SER-1 is potentially involved in the control of head muscle. In addition, we have identified the G-protein signaling pathways involved in the monoaminergic modulation of the ASHs and have demonstrated that neuropeptides encoded by *nlp-3* are essential for the serotonergic stimulation of the ASH-mediated locomotory circuit. These results highlight the utility of the *C. elegans* model for study of monoaminergic/peptidergic interactions in individual neurons of complex, sensory-mediated circuits.
Objectives

Serotonin (5-HT) modulates a number of key behaviors in *Caenorhabditis elegans*, but our understanding of the mechanisms underlying serotonergic modulation, including the 5-HT receptors and their downstream effectors is clearly still in its infancy. I propose to use the well characterized and genetically-manipulable, *C. elegans* model system to examine the serotonergic modulation of simple, sensory-mediated locomotory behaviors, with the goal of understanding how serotonergic signaling modulates neuronal activity state. It is anticipated that observations in *C. elegans* will be useful in understanding the more complex and, in many cases, experimentally-intractable, serotonergic circuits operating in mammals that are involved in altered behavioral states, such as anxiety, depression and obesity. 5-HT regulates an array of simple and complex processes in *C. elegans* and translates nutritional status into distinct locomotory states. I will characterize how serotonergic and peptidergic signaling translate nutritional state into fine tuning sensory mediated locomotory behaviors using reverse genetics, neuron selective gene expression and RNAi strategies.

Specifically, I will

1) Characterize the role of 5-HT receptors in 5-HT dependent increases in aversive behavior.

2) Characterize the role of 5-HT in the modulation of the glutamatergic/peptidergic ASH sensory neurons.

3) Characterize the role of various classes of serotonergic neurons in food-dependent increases in aversive behavior.
2.0 CHAPTER II

Materials and Methods

2.1 Materials. All reagents were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco’s Modified Eagles Medium (DMEM) was purchased from Media-Tech (Herdon, VA), neurochemicals from Sigma-Aldrich (St. Louis, MO), restriction enzymes from New England Biolabs (Beverly, MA) and Promega (Madison, WI) and oligonucleotide primers from Integrated DNA Technologies (Coralville, IA). A C. elegans cDNA pool was purchased from OriGene Technologies (Rockville, MD), and additional cDNA pools were constructed from mixed stage mRNA using standard techniques. Green fluorescent protein (GFP) expression vectors were obtained from Andy Fire (Stanford School of Medicine).

2.2 Cultures and maintenance of strains. The N2 Bristol WT isolate of C. elegans was used for all studies. All animals were raised at 20°C under uncrowded conditions (Brenner, 1974). The following mutant alleles were used in this study: acy-1(ce2)III, dgk-1(sy428)X, eat-4(ky5)III, eat-16(ce71)I, egl-3(n150)V, egl-8(n488)V, egl-21(n476)IV, egl-30(js126)I, eri-1(kp3948)IV, gsa-1(ce81)I, ins-1(tm1888)IV, mod-5(n822)I, octr-1(f14d12.6)(ok371)X, nlp-3(tm2302)X, pde-4(ce268)II, rrf-3(pk1269)II, ser-5(tm2647)I, ser-5(tm2654)I, ser-4(ok512)III, mod-1(ok103)V, ser-7(tm1325)X, ser-
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1(ok345)X, ttx-3(ks5). All strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) except, nlp-3(tm2302)X, ser-7(tm1325), ser-5(tm2647), ser-5(tm2654), which were received from the National Bio-Resources Project (Tokyo Women’s Medical University, Tokyo, Japan). tph-1(mg280) animals were provided by Dr. Mark Alkema (MIT, Boston, MA), ttx-3p::mod-1(+), odr-2(2b)p::mod-1(+), ceh-2p::tph-1(+) and srh-142p::tph1(+) animals from Dr. Cori Bargmann (Rockefeller University, New York, NY; Zhang et al., 2005), ser-1(ok345); mod-1(ok103) animals from Dr. Ji Ying Sze (Albert Einstein College of Medicine, New York, NY) and glr-3p::ICE animals from Dr. Villu Maricq (University of Utah, Salt Lake City, UT). Full-length egl-3p::egl-3(+) rescued animals were kindly provided by Dr. Maureen Barr (Rutgers University, Piscataway, NJ). ins-1 overexpressors (ins-1p::ins-1XS) were kindly provided by Dr Yuichi Iino (The University of Tokyo, Tokyo, Japan). Animals containing combinations of null or gf alleles were constructed using standard genetic techniques and confirmed by PCR. All mutant animals were backcrossed with the N2 Bristol strain at least 4X before use.

2.3 Behavioral assays. Assay plates (5 cm NGM plates) were prepared daily and serotonin (4 mM) was added to NGM liquid media just prior to pouring. Dilute 1-octanol was prepared daily using 100% ethanol (vol/vol) (Sulston and Hodgkin, 1988; Bargmann et al., 1993; Chao et al., 2004). Synchronized fourth-stage larvae (L4) were picked 24 hrs pre-assay and assays were performed at 23-25°C. Octanol avoidance was measured as
described by Chao et al., 2004. Briefly, the blunt end of a hair (Loew-Cornell 9000 Kolinsky 8 paintbrush), taped to a toothpick, was dipped in 30% octanol and the hair was placed in front of an animal exhibiting forward sinusoidal locomotion. Time to reverse was recorded and assays were terminated after 20 sec, as wild-type animals spontaneously reverse on average every 20 sec (Zhao et al., 2003; Chao et al., 2004; Chao et al., 2005). For assays in the absence of food or exogenous 5-HT, well-fed young adults (three to five per plate) were first transferred to intermediate non-seeded plates and left for 1 min to prevent any bacteria/media carry over, then transferred to NGM plates and assayed after 10 min. For assays in the presence of food (E. coli OP50) or 5-HT, animals were transferred to plates containing a thin layer of OP50 or 4 mM 5-HT and assayed after 20 and 30 min, respectively.

Reversal frequency was assayed as described previously (Tsalik and Hobert, 2003; Dernovici et al., 2006). Well-fed animals were transferred to NGM plates for 30 sec, then transferred to assay plates (+/- food) for 1 min and assayed. Reversal frequency was scored as the number of times an animal reversed within 3 min (Pierce-Shimomura et al., 1999). Data was presented as a mean +/- SE (n = 3) and analyzed by 2-tailed Student’s t test. P values were indicated as follows: *, P < 0.05, **, P < 0.01 ***, P < 0.001.

Post-initiation assay was performed as follows: Synchronized fourth-stage larvae (L4) were picked 24 hrs pre-assay and assays were performed at 23-25°C. Octanol avoidance was measured as described by Chao et al., 2004. First, animals were examined for duration of reversal per acute response to dilute octanol, by counting the number of head swings per reversal. Second, the angle turned away from the initial trajectory upon
encountering octanol was also measured. Angles turned were categorized as follows: turning 0° (return in the same path), 0-45°, 45-90° or 90-180° (omega turn). 10 animals were examined per strain per condition. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001.

2.4 RNA interference. RNA interference (RNAi) was performed as described previously using wild-type N2, rrf-3(pk1629) or eri-1(kp3948) (Kamath and Ahringer, 2003). Hermaphrodites and their progeny were grown on NGM plates containing 25 µg carbenicillin and 1 mM isopropyl-β-D-thiogalactopyranosidase (IPTG) and were seeded with HT115 (DE3) bacteria containing ser-5 encoded RNAi vector or empty vector. Synchronized L4s were picked 24 hours pre-assay and examined for octanol sensitivity. The following RNAi animals were generated through feeding: ser-5RNAi.

2.5 Rescue constructs and strains. All rescue constructs were created by overlap fusion PCR or by cloning into pPD95.75 (Mello and Fire, 1995; Hobert, 2002). For overlap PCR, constructs were pooled from at least 3 reactions and were co-injected with myo-3p::gfp, F25B3.3p::gfp or rol-6 and carrier DNA (to 100 ng) into gonads of wild-type and null mutant animals by standard techniques (Kramer et al., 1990; Mello and Fire, 1995). At least three lines expressing each construct were examined. All constructs in pPD95.75 were confirmed by sequencing.

FY748 mod-1(ok103)grEx160[mod-1p::mod-1] expresses a full-length mod-1 transgene in mod-1 null animals. The mod-1 transgene includes a 2 kb mod-1 promoter, the mod-1 open reading frame and 706 bp of the mod-1 3′UTR and was created as
described by (Carnell et al., 2005). FY751 *ser-5*(tm2654)*grEx163*[sra-6p::ser-5::gfp]* expresses SER-5 in the ASH sensory neurons of *ser-5* null animals. The *sra-6p::ser-5::gfp* transgene includes the *sra-6* promoter, the *ser-5* cDNA, sequence coding for GFP and the *unc-54* 3’UTR. FY750 *ser-5*(tm2654)*grEx162*[ser-5p::ser-5]* expresses a full-length *ser-5* transgene in *ser-5* null animals. RWK8 *mod-1*(ok103)*fvEx3*[npr-9p::mod-1::gfp]* expresses MOD-1 in the AIB interneurons of *mod-1* null animals. The *npr-9p::mod-1::gfp* transgene includes 2 kb of the *npr-9* promoter, the *mod-1* cDNA, sequence coding for GFP and the *unc-54* 3’ UTR. The full-length genomic *nlp-3* transgene includes a 3 kb *nlp-3* promoter, the *nlp-3* open reading frame and 618 bp of the *nlp-3* 3’UTR. The full-length *eat-4* genomic transgene includes a 2 kb *eat-4* promoter, the *eat-4* open reading frame and 1.3 kb of the *eat-4* 3’UTR. The full-length genomic *egl-21* transgene includes a 1.3 kb *egl-21* promoter, the *egl-21* open reading frame and 2.2 kb of the *egl-21* 3’UTR. A full-length genomic *ser-5* transgene includes a 5 kb promoter, the *ser-5* reading frame and 1 kb of *ser-5* 3’UTR. All ASH-selective transgenes express the relevant cDNAs under the control of a 3.5 kb ASH selective promoter (*sra-6p*, Troemel et al., 1995). All NSM selective transgenes express the relevant cDNAs under the control of an NSM selective promoter (*ceh-2p*, 1.6 kb promoter, Aspock et al., 2003). All ADF specific transgenes express the relevant cDNAs under the control of an ADF selective promoter (*srh-142p*, 1.6 kb, Sagasti et al., 1999). At least 3 products were pooled and microinjected at 1-40 ng/µL, with 30 ng of *myo-3p::gfp* or *F25B3.3p::gfp* marker and at least 3 transgenic lines were examined (Kramer et al., 1990; Mello and Fire, 1995).
2.6 Localization of SER-5 expression. RWK7 \textit{ser-5(tm2654)fjEx2[ser-5p::ser-5::gfp]} expresses a full-length \textit{ser-5} translational fusion from the 5 kb \textit{ser-5} promoter that includes the endogenous \textit{ser-5} 3’ UTR with sequence encoding GFP inserted into the predicted \textit{SER-5} C-terminus 10 aa after TMVII. The \textit{ser-5::gfp} transgene was created by overlap fusion PCR from three different products fused by two rounds of PCR. Primers amplified the \textit{ser-5} 5’-end ~5 kb upstream of the ATG up to a portion of exon 6 using SER-5F1 and SER-5R+GFP1. A second set of primers (SER-5F+GFP2 and SER-5R2) amplified a short \textit{gfp} overlap with the \textit{ser-5} 3’ end including the predicted \textit{SER-5} C-terminus 10 aa after TMVII and the \textit{ser-5} 3’UTR, as follows. Sequence encoding \textit{gfp} was amplified using GFPF3b and GFPR3 and then fused in frame with the \textit{ser-5} 3’ product (using GFPF4 and SER-5R4) and this product was then combined with the \textit{ser-5} 5’ fragment to generate a full-length \textit{ser-5} transgene that included sequence coding for GFP inserted in the predicted C terminus of the receptor using nested primers. PCR products were pooled from at least 3 separate reactions (5 ng total) and were coinjected either alone or with \textit{rol-6} plasmid (pRF4) and carrier DNA into gonads of \textit{ser-5(tm2564)} null animals by standard techniques (Mello and Fire, 1995). Uptake of DiD in living animals was assayed as described (Herman and Hedgecock, 1990). Briefly, stock solution (1 mM) of 1’, 1-dioctadecyl-3, 3, 3-tetramethylindocarbocyanine (DiD; Invitrogen/Molecular Probes, Eugene, OR) was diluted 1:200 in M9 buffer. Animals were incubated in 100 \textmu l of diluted DiD for 1 hr at RT, transferred to a fresh NGM plate seeded with OP50 and allowed to crawl on the bacterial lawn for 1–2 hrs to destain and were then placed on agarose pads with 20 mM sodium azide for visualization. At least
five transformed lines were analyzed for \textit{gfp} fluorescence and DiD staining using an Olympus confocal microscope.

\textbf{2.6 (ii) Multiple sequence alignments and production of phylogenetic trees.} To remove hypervariable regions, each vertebrate and invertebrate 5-HT receptor sequence was truncated at the N-terminus (three residues before TMI), the third intracellular loop (eight residues after TMV and six residues before TMVI), and the C-terminus (twelve residues after TMVII). The initial alignment was performed with the modified sequences using the MegAlign program (DNASTar, Madison, WI) with ClustalW parameters set to optimize protein sequence alignments (Multiple Alignment Parameters: Gap opening penalty of 15, and Gap extension penalty of 0.3; Pairwise Alignment Parameters: Gap opening penalty of 35, and Gap extension penalty of 0.75). The resulting alignment was then fine-tuned manually. All alignments are available upon request. Bootstrapping was also performed with DNASTar (1000 replicates, random seed), and the resulting tree was then imported into the Phylogenic Alignment Utility Program (PAUP, version 4.0b10), where it was analyzed under the distance, Neighbor-Joining method to produce the tree reported here. The \textit{Caenorhabditis elegans} GAR-1 sequence was utilized as an outgroup for analysis of 5-HT receptors. Accession numbers for all sequences used are listed in the legend to Fig. (6).

\textbf{2.7 Creation of combination mutant animals.} Animals containing combinations of \textit{gf}, \textit{lf} or null alleles were constructed using standard genetic techniques and confirmed by PCR. \textit{ser-5(tm2654)I} animals were crossed with either \textit{egl-30(js126)I}, \textit{gsa-1(ce81)I} or \textit{pde-
4(ce268)II animals to create egl-30(js126) ser-5(tm2654)I (RWK94), gsa-1(ce81) ser-5(tm2654)I (RWK95) and ser-5(tm2654)I;pde-4(ce268)II (RWK96) double mutants. nlp-3(tm2302)X animals were crossed with pde-4(ce268)II, egl-30(js126)I or gsa-1(ce268)I animals to give pde-4(ce268)II; nlp-3(tm2302)X (RWK97), gsa-1(ce81)I; nlp-3(tm2302)X (RWK104) and egl-30(js126)I; nlp-3(tm2302)X (RWK98) double mutants, respectively. eat-4(ky5)V animals were crossed with ser-5(tm2654)I to create ser-5(tm2654)I; eat-4(ky5)V (RWK101). eat-4(ky5)V animals were also crossed with egl-3(n150)I; nlp-3(tm2302)X to generate eat-4(ky5)I;egl-3(n150)I (RWK102) and eat-4(ky5)V;nlp-3(tm3202)X (RWK103) double mutants, respectively. All mutant animals were screened by PCR with primers flanking the deletions to confirm the crosses. To confirm that the mutant animals were homozygous for the deletion, a second PCR reaction was conducted using the forward primer flanking the deletion and a reverse primer internal for the deletion. Homozygous mutants produced no PCR product in this reaction. All mutant animals were backcrossed with the N2 Bristol strain at least 4X before use in assays or crosses. The following strains were backcrossed with N2 Bristol strain before use in the generation of the double mutants, eat-4(ky5)4X (RWK22), egl-3(n150)4X (RWK23), nlp-3(tm2302)4X (RWK24) and ser-5(tm2654)5X (RWK51).

2.8 Creation of cell specific RNAi constructs. Neuron selective/specific RNAi transgenes were constructed as described by Esposito et al., 2007 (Sense and antisense primers and a list of strains, are available below in Materials and Methods). For the
creation of RNAi transgenes, a neuron-selective promoter (*sra-6p* for ASH/ASI; *gpa-4p* for ASI) was fused to exon rich regions of the target gene. Exon rich regions were amplified using a forward and reverse primer to create template A from either cDNA or genomic DNA by PCR-fusion, as previously described (Hobert, 2002). Neuron-selective promoters were amplified using a forward primer with reverse (sense) or reverse (antisense) primers to create templates B and C, respectively (see Esposito et al., 2007). Templates A and B were then fused using a forward internal promoter primer and a reverse internal target gene primer to create the sense construct (Product D). Templates A and C were fused using the forward internal promoter primer and the forward internal target gene primer to create the antisense construct (Product E). At least 3 products were pooled and sense and antisense transgenes were microinjected at 25-100 ng/µL, with 30 ng of *myo-3p::gfp* or *F25B3.3p::gfp*. Multiple transgenic lines were examined for each RNAi.

All ASH-selective RNAi strains were generated by expressing RNAi under the control of 3.5 kb of an ASH-selective promoter (*sra-6p*; Troemel et al., 1995). ASI-specific RNAi strains were generated by expressing RNAi under the control of 2.8 kb of an ASI-specific promoter (*gpa-4p*; Jansen et al., 1999). Neuron-selective RNAi transgenes were injected into either N2 or mutant animals. ADF, NSM, AWB and RIA specific RNAi strains were generated by expressing RNAi under the control of *srh-142*, *ceh-2*, *str-1* or *glr-3* promoters, respectively (Troemel et al., 1997; Sagasti et al., 1999; Aspock et al., 2003; Mukhopadhyay et al., 2007).
3.0 CHAPTER III

Three distinct amine receptors operating at different levels within the ASH locomotory circuit are each essential for the serotonergic modulation of chemosensation in Caenorhabditis elegans


3.1 Results

SER-1 and MOD-1 are each essential in modulating sensitivity to dilute octanol. Wild-type animals on food (E. coli OP50) or incubated in exogenous 5-HT (4 mM) exhibit dramatically increased aversive responses to dilute octanol, but the serotonergic receptors and pathways mediating these effects have not been identified (Chao et al., 2004). C. elegans contains at least four previously characterized 5-HT receptors: three G-protein coupled receptors, SER-1, SER-4 and SER-7, and a novel 5-HT gated Cl⁻ channel, MOD-1 (Olde and McCombie, 1997; Hamdan et al., 1999; Ranganathan et al., 2000; Hobson et al., 2003). Animals carrying null alleles for each of these 5-HT receptors have been characterized in other behavioral contexts (Ranganathan
et al., 2000; Hobson et al., 2003; Dempsey et al., 2005; Zhang et al., 2005). To identify the individual 5-HT receptor(s) and neurons involved in the serotonergic modulation of octanol avoidance, we examined the responses of each of these 5-HT receptor null mutants to dilute (30%) octanol, a behavior mediated by the ASH sensory neurons (Chao et al., 2004). The responses of these mutant animals to dilute octanol were identical to those of wild-type animals either off food or in the absence of exogenous 5-HT (Fig. 2). However, both the ser-1 and mod-1 null animals failed to increase their sensitivity to dilute octanol on food or in the presence of exogenous 5-HT, in contrast to ser-7, ser-4 or wild-type animals (Fig. 2). The role of mod-1 and ser-1 in 5-HT sensitivity was confirmed by transgenic rescue, i.e., the expression of full-length mod-1 or ser-1 transgenes in mod-1 and ser-1 null animals, respectively, restored 5-HT sensitivity. As predicted, ser-4; mod-1; ser-7 ser-1 quadruple null animals that lack all previously identified 5-HT receptors also failed to increase sensitivity to dilute octanol in the presence of either food or 5-HT (Fig. 2). These results suggest that both ser-1 and mod-1, but not ser-4 or ser-7, have essential roles in the modulation of food and 5-HT dependent increases in sensitivity to dilute octanol.
Figure 2. Food and 5-HT dependent increases in sensitivity to dilute octanol require both MOD-1 and SER-1. Wild-type and 5-HT receptor null mutant animals were examined for their ability to respond to dilute (30%) octanol in the presence or absence of food (E. coli OP50) or 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by a two-tailed Student’s t test. “***”, P< 0.001, indicates significantly different from wild-type animals under identical test conditions. n value under each bar represents the number of animals examined per strain. Quad, ser-4(ok512); mod-1(ok103); ser-7(tm1325) ser-1(ok345); Quint, ser-5(tm2654); ser-4(ok512); mod-1(ok103); ser-7(tm1325) ser-1(ok345).
MOD-1 and SER-1 function in the AIB or AIY and the RIA interneurons, respectively, to modulate ASH-mediated aversive responses to dilute octanol.

The neuronal expression of mod-1 and ser-1 has been characterized previously using various mod-1p::gfp and ser-1p::gfp reporter constructs. mod-1p::gfp transcriptional fusions are expressed in the AIA, AIB, AIY, AIZ, and RID interneurons, as well as a number of additional unidentified neurons in the head, ventral cord and tail. While ser-1p::gfp constructs depending on the ser-1::gfp transgene exhibit variable expression, in pharyngeal and vulval muscles, as well as a number of neurons in the head and tail, including the RIA interneurons and the ring and ventral cord motor neurons (Ranganathan et al., 2000; Tsalik et al., 2003; Wenick and Hobert, 2004; Carnell et al., 2005; Dempsey et al., 2005; Xiao et al., 2006; Dernovici et al., 2007).

To identify the neurons involved in the MOD-1-mediated modulation of ASH-mediated aversive responses, we examined the 5-HT sensitivity of aversive responses to dilute octanol in mod-1 null animals expressing ttx-3p::mod-1 or odr-2(2b)p::mod-1 transgenes previously observed to rescue the 5-HT sensitivity of aversive olfactory learning in mod-1 null animals (Zhang et al., 2005). The ttx-3 promoter appears to be expressed exclusively in the AIY interneurons in adult animals, while odr-2(2b)p drives expression primarily in the AIB and AIZ interneurons (Chou et al., 2001; Hobert et al., 1997; Wenick and Hobert, 2004; Bendena et al., 2008). In addition, we also examined mod-1 null animals expressing an npr-9p::mod-1 transgene that is expressed almost exclusively in the AIB interneurons (Bendena et al., 2008). All three transgenes restored 5-HT sensitivity in mod-1 null animals (Fig. 3A). Interestingly, ttx-3 null animals in which the AIYs fail to develop exhibited wild-type responses to dilute octanol both on
and off food (Hobert et al., 1997, Fig. 3A). However, since mod-1 encodes an inhibitory Cl⁻ channel, animals that lack AIYs, such as the ttx-3 null animals or animals in which the AIYs are inhibited, as in the mod-1 expressing animals, would both be predicted to increase aversive responses to dilute octanol in the presence of food or 5-HT, exactly as we observed in the present studies. Together, these results suggest that the AIB and AIY interneurons are involved in modulating the 5-HT sensitivity to dilute octanol.

As noted above, the 5-HT sensitivity of ser-1 null animals was rescued with a full-length ser-1p::ser-1::gfp transgene (Fig. 4A). This ser-1p::ser-1::gfp transgene contains a truncated 3.6 kb promoter and is expressed in the RIA interneurons and many of the ring motor neurons, major downstream partners of the RIAs, but apparently not in the ventral cord motor neurons, as noted for other ser-1p::gfp transgenes (Carnell et al., 2005; Xiao et al., 2006; Dernovici et al., 2007). Therefore, since the ASHs innervate the RIAs directly, we examined the potential role of this RIAs/ring motor neuron pathway in the modulation of serotonergic signaling mediating aversive behavior, using wild-type animals expressing either a glr-3p::ICE transgene that selectively ablates the RIAs or a glr-3p::ser-1(RNAi) transgene that should specifically knockdown ser-1 expression in the RIAs (Fig. 4A). Animals expressing either transgene exhibited wild-type responses to dilute octanol off food, suggesting that the pathways sensing dilute octanol were intact (data not shown). In contrast, both animals failed to increase octanol sensitivity when incubated in 5-HT, further supporting a role for the RIAs in the SER-1-mediated serotonergic modulation of olfaction (Fig. 4A).

Together, these results suggest that mod-1 functions in the AIB and AIY interneurons and ser-1 functions in the RIA/ring motor neuron pathway that ultimately
innervates primarily head muscle in the serotonergic sensitization of aversive responses to dilute octanol (White et al., 1986; Alkema et al., 2005).
Figure 3. Rescue of 5-HT dependent increases in aversive responses to dilute octanol and food-dependent alterations in the rate of spontaneous reversals in mod-1 null animals. A. Wild-type or mutant animals expressing the indicated transgenes were assayed for 5-HT dependent increases in aversive responses to dilute (30%) octanol, as described in Methods. B and C. Spontaneous reversals (reversals/3 min) were quantified in well-fed animals 1 min after transfer to plates without (B) or with E. coli OP50 (C). Data are presented as a mean ± SE and analyzed by a two-tailed Student’s t test. “***” P < 0.001, indicates significantly different from wild-type animals under identical test conditions. The results from rescued mod-1 null animals are indicated by hatched bars.
Figure 4. Rescue of 5-HT dependent increases in aversive responses to dilute octanol and food-dependent alterations in the rate of spontaneous reversals in ser-1 null animals.  

A. Wild-type or mutant animals expressing the indicated transgenes were assayed for 5-HT dependent increases in aversive responses to dilute (30%) octanol as described in Methods.  

B and C. Spontaneous reversals (reversals/3 min) were quantified in well-fed animals 1 min after transfer to plates without (B) or with *E. coli* OP50 (C).  

Data are presented as a mean ± SE and analyzed by a two-tailed Student’s *t* test. “***” *P* < 0.001, significantly different from wild-type animals under identical test conditions.  

Results from rescued *ser-1* null animals are indicated by hatched bars.
The expression of SER-5 in the ASH sensory neurons is essential for the serotonergic modulation of aversive responses to dilute octanol.

As noted previously, the ASH sensory neurons are necessary and sufficient for responses to dilute octanol and 5-HT increases calcium transients in the ASH sensory neurons, suggesting that a 5-HT receptor may be expressed directly in the ASHs (Chao et al., 2004). However, none of the previously characterized 5-HT receptors appears to be expressed in the ASHs, suggesting that an additional 5-HT receptor might remain to be identified. To address this possibility, RNAi knockdown of predicted, previously uncharacterized, *C. elegans* biogenic amine receptors (*tyra-3, f14d12.6, f16d3.7(ser-5)*) was used to screen for additional GPCRs that might be involved in 5-HT dependent increases in octanol sensitivity in both wild-type and RNAi-sensitive, *rrf-3(pk1269)*, strains (Simmer et al., 2002). As noted in Fig. 5A, *ser-5(RNAi)* completely abolished 5-HT dependent increases in octanol sensitivity in the *rrf-3*, but not wild-type animals. Consistent with these RNAi results, *ser-5(tm2654)* and *ser-5(tm2647)* animals exhibited wild-type responses to dilute octanol, but also failed to increase octanol sensitivity in the presence of 5-HT (Fig. 5B). *ser-5(tm2654)* animals contain a 348 bp deletion in exon 4 resulting in a deletion of a portion of TM5, the 3rd intracellular loop and half of TM6. *ser-5(tm2647)* animals contain a 219 bp deletion in exon 4, resulting in a deletion of a portion of TM4, the 3rd extracellular loop, TM5 and a portion of the 3rd intracellular loop. Therefore, *ser-5(tm2654)* and *ser-5(tm2647)* are predicted null alleles, as TMs 5 and 6 are required for ligand-binding (Strader et al., 1989; Choudhary et al., 1993; Almaula et al., 1996; Roth et al., 1997) and G-protein coupling (Moro et al., 1993).
As anticipated, food and 5-HT dependent increases in responses to dilute octanol could be restored in ser-5 null animals by the expression of a full-length ser-5 transgene (Fig. 5B). In addition, 5-HT sensitivity in ser-5 null animals also could be rescued with a sra-6p::ser-5 transgene whose expression is confined primarily to the ASHs, suggesting that SER-5 may function directly in the ASHs (Fig. 5B; Troemel et al., 1995). Our analysis of ser-5 expression from ser-5p::gfp transcriptional and translational fusions agrees with data reported previously, i.e., fluorescence was observed in vulval and body wall muscle, as well as a number of neurons in the head and tail (Fig. 6C; Wormbase.org; Carre-Pierrat et al., 2006). GFP fluorescence also was observed in the AWB sensory neurons, and more faintly and variably in the ASHs (Fig. 6C). Low levels of SER-5 expression in the ASHs may be sufficient to confer 5-HT sensitivity and/or, alternatively, SER-5 might be acting at some other site in the serotonergic circuit. Therefore, to confirm a role for SER-5 directly in the ASHs we examined the 5-HT sensitivity of aversive responses to dilute octanol in wild-type animals in which SER-5 expression in the ASHs was knocked down by the expression of a sra-6p::ser-5(RNAi) transgene. As predicted, these animals exhibited a wild-type response to dilute octanol, but failed to increase octanol sensitivity in response to 5-HT, as observed above for the two ser-5 null mutants (Fig. 5B). Together, these results suggest that SER-5 functions in the ASHs and increases their responsiveness to dilute octanol. Whether SER-5 increases the sensitivity of the putative octanol receptors, downstream signaling and/or neurotransmitter release remains to be determined.

No ser-5 ESTs are present in the database and during the generation of a full-length ser-5 cDNA for expression studies using 5’ and 3’ RACE, we observed that the predicted
ser-5 intron/exon splicing pattern and ser-5 cDNA differed significantly from that published in Wormbase.org (Hapiak et al., 2009). As suggested from the behavioral analyses described above, the new predicted SER-5 amino acid sequence is most closely related to mammalian 5-HT₆ receptors (Fig. 6A). For example, SER-5 was 35% identical to the human 5-HT₆ receptor within predicted transmembrane domains involved in ligand binding. In addition, both SER-5 and 5-HT₆ have short 3rd intracellular loops (67 and 60 aas, respectively) with BBXXB₂⁹₆ (where B is a basic amino acid) motifs at the C-termini of the loop that in other GPCRs can confer constitutive activity and a conserved PLRYK₁⁶⁹ motif in the second intracellular loop that is not found in any other 5-HT receptors.

Finally, the genes encoding the mammalian 5-HT₆ receptors contain two introns within sequence encoding the putative third intracellular and third extra-cellular loops. The positions of both introns are conserved in ser-5 (Hapiak et al., 2009). Interestingly, all of the previously characterized 5-HT receptors clustered according to their documented G-protein coupling, regardless of phylogenetic origin, validating the utility of the tree and supporting the identification of SER-5 as a Gₛ-coupled 5-HT receptor (Fig. 6B).

Although this tree was generated using truncated amino acid sequences with the more variable N and C termini and third intracellular loops deleted, similar results were obtained using the full-length receptors (data not shown). Interestingly, during lethargus mutations that increase Gₛ signaling dramatically increase aversive responses to dilute octanol in the absence of food or exogenous 5-HT (Raizen et al., 2008). We have observed similar effects in wild-type animals (Harris, unpublished). It will be important to determine if SER-5 and/or the ASH sensory neurons are essential for this Gₛ-mediated increase in octanol sensitivity or lethargus.
Figure 5. **SER-5 is essential for 5-HT dependent increases in sensitivity to dilute octanol.**

**A.** Effect of *ser-5* RNAi on sensitivity to dilute (30%) octanol in both wild-type and *rrf-3(pk1269)* animals. **B.** Wild-type and *ser-5* null animals were examined for aversive responses to dilute octanol in the presence or absence of 5-HT (4 mM), as described in Methods. In addition, *ser-5* expression in the ASH sensory neurons was knocked down in wild-type animals (*sra-6p::ser-5(RNAi)* animals). Data are presented as a mean ± SE and analyzed by two-tailed Student’s *t* test. “***” *P* < 0.001, significantly different from wild-type or *rrf-3(pk1269)* animals under identical test conditions. The results from rescued *ser-5* null animals are indicated by hatched bars.
Figure 6. SER-5 is most identical to invertebrate/mammalian 5-HT receptors and is expressed in both neurons and muscle. A. Predicted amino acid sequence of SER-5.

The full-length ser-5 cDNA sequence was generated by 5’ and 3’ RACE as described in Methods. Putative transmembrane regions are shaded in grey. Residues potentially involved in BA binding are in white and G-protein coupling residues are underlined (Strader et al., 1989; Choudhary et al., 1993; Moro et al., 1993; Barak et al., 1994; Almaula, 1996; Roth et al., 1997). Potential PKA and PKC phosphorylation sites were identified using ScanProsite, and are indicated in bold and grey, respectively (Gatticker et al., 1992; Lymbery et al., 1993; Strader et al., 1989; Choudhary et al., 1993; Moro et al., 1993; Barak et al., 1994; Almaula, 1996; Roth et al., 1997).
Sequestriation and desensitization sites are bold and underlined (Barak et al., 1994). B. Unrooted phylogenetic tree of vertebrate and invertebrate 5-HT receptors.

Sequences were modified to remove hypervariable regions by the deletion of the N-termini three aas before the first predicted transmembrane domains, the third intracellular loops, eight aas after and six aas before predicted transmembrane domains five and six, respectively, and the C-termini, 12 aas following predicted transmembrane domain seven. Annotated sequences were initially aligned using MegAlign in DNAStar with Clustal W, and using parameters defined in methods and fine-tuned by hand. Bootstrapping was undertaken in DNAStar (1000 replicates with random seed). *Homo sapiens* (H) in green: H5HT-1a (CAA40962), H5HT1b (P28222), H5HT1d (P28221), H5HT1e (CAA77558), H5HT1f (AAA36605), H5HT2a (CAA40963), H5HT2b (CAA54513), H5HT2c (AAF35842), H5HT4 (Q13639), H5HT5a (CAA57168), H5HT6 (AAA92622), Hs5HT7 (CAH69965), *Caenorhabditis elegans* (Ce) in red: CeSER1 (NP_001024728), CeSER4 (NP_497452), CeSER7 (NP_741730), CeSER5 (in manuscript), *Caenorhabditis briggsae* (Cb): CbSER-1 (CAE69959), CbSER4 (CAE69091), CbSER7 (CAE58847), CbSER5 (CAE60436), *Aedes egyptii* (Ae): Ae5HT7 (AAG49292), *Drosophila melanogaster* (Dm) in blue: Dm5HT7 (NP_524599), Dm5HT2a (NP_725849), Dm5HT2b (CAA77571), Dm5HT (NP_730859), *Haemonchus contortus* (Hc): Hc5HT1e (AAO45883), *Ascaris suum* (As): As5HT-2c (AAC78396). C. GFP fluorescence from a full-length *ser-5p::ser-5::gfp* transgene that includes sequence coding for GFP inserted into the predicted C-terminus of the receptor. A. merge of GFP fluorescence and DID staining in
the nerve ring, B. Single Z-section of GFP/DID merge in the nerve ring. C. and D. Inset from (B) with GFP fluorescence (C) and DID staining (D).
The serotonergic receptors and pathways modulating ASH-mediated aversive behaviors overlap with those modulating the overall rate of spontaneous reversals.

The spontaneous initiation of backward locomotion in *C. elegans* is regulated by the presence of food and 5-HT (Tsalik and Hobert, 2003, Wakabayashi et al., 2004; Gray et al., 2005; Dernovici et al., 2007). For example, in wild-type animals, 5-HT decreases the rate of spontaneous reversal, based on the observation that *tph-1(mg280)* null animals that lack detectable 5-HT and animals in which the serotonergic neurons have been ablated exhibit a markedly decreased duration of forward movement, i.e., they reverse more frequently (Wakabayashi et al., 2004). In contrast, 5-HT markedly decreases the time taken to reverse in response to dilute octanol (Chao et al., 2004; present study). Therefore, to better understand these two potentially conflicting observations, we examined the rate of spontaneous reversal in the presence of food and after 1 min in the absence of food in both the mutant and rescued animals used in the present study (Figs. 3 and 4). As noted by others, wild-type animals reverse more frequently immediately off food compared to on food (Figs. 3 and 4; Tsalik and Hobert, 2003). This is also true for *ser-1, mod-1, ser-5* and *ser-4;mod-1;ser-7 ser-1* null animals (Figs. 3 and 4; data not shown, Dernovici et al., 2007). However, *ser-1* or *mod-1* null animals reverse significantly more frequently than wild-type animals off food (Figs. 3B and 4B) and significantly less frequently than wild-type animals on food (Figs. 3C and 4C). The decreased reversal frequencies of the *ser-1* and *mod-1* null animals on food contrast sharply with those of *tph-1* null animals that lack 5-HT or *ser-4;mod-1;ser-7 ser-1*.
animals that lack most serotonergic signaling, i.e., both tph-1 and ser-4;mod-1;ser-7 ser-1 null animals reverse significantly more frequently than wild-type animals on food.

As noted above, the expression of mod-1 in either the AIYs (ttx-3p), AIBs/AIZs (odr2(2b)p) or AIBs (npr-9p) interneurons was sufficient to rescue the 5-HT dependent stimulation of aversive responses to dilute octanol in mod-1 null animals, suggesting that the AIBs and probably AIYs play essential roles in modulating the 5-HT sensitivity to dilute octanol (Fig. 4). In contrast, as shown in Fig. 3, the expression of mod-1 in the AIBs or AIBs/AIZs of mod-1 null animals on food or in the AIBs of mod-1 null animals off food did not restore the rate of spontaneous reversals to wild-type levels. The rate of spontaneous reversal was only rescued to wild-type levels in animals expressing mod-1 in the AIYs on food or the AIYs and AIBs/AIZs off food (Figs. 3B and C). Together these results suggest that MOD-1 plays different roles in modulating the rate of spontaneous reversal and ASH-mediated aversive responses and suggest a potential role for the AIZ interneurons in modulating the rate of spontaneous reversal immediately off food.

The ser-1 transgene used to rescue the 5-HT sensitivity of aversive responses to dilute octanol in ser-1 null animals also restored the rate of spontaneous reversal to wild-type levels both on and immediately off food (Figs. 4B and 4C). This ser-1 transgene appears to be expressed in the RIA and a number of ring motor/interneurons (Xiao et al., 2006; Dernovici et al., 2007). Interestingly, animals lacking RIAs (glr-3p::ICE) or with reduced SER-1 expression in the RIAs (glr-3p::ser-1(RNAi)) failed to increase aversive responses to dilute octanol in presence of 5-HT (Fig. 4A), but still exhibited wild-type
rates of spontaneous reversal both on and immediately off food (Figs. 4B and C; Tsalik and Hobert, 2003). These results highlight the importance of the RIAs in the serotonergic sensitization of aversive responses to dilute octanol and suggest that other neurons, possibly the ring motor neurons, are responsible for SER-1 mediated modulation of spontaneous reversal.

Together, these results highlight the complexity of serotonergic signaling in the modulation of reversal responses and suggest that ser-1 and mod-1 inhibit reversal off food and stimulate reversal on food, in agreement with their proposed stimulatory roles in mediating the food and 5-HT dependent increases in aversive responses to dilute octanol observed above.
3.2 Discussion

In the present study, we have identified three amine receptors that are each essential for 5-HT dependent increases in aversive responses to dilute octanol. Off food, wild-type, and 5-HT receptor mutants respond identically to dilute octanol. In contrast, on food or in the presence of 5-HT, wild-type animals respond more rapidly and this increased aversive response is completely abolished in animals lacking either SER-5, MOD-1, or SER-1. Interestingly, no intermediate responses were observed; each of the receptors appear to be essential for 5-HT sensitization, suggesting that each plays a different, but critical role in the process.

Serotonergic signaling enhances responses to dilute octanol at multiple levels within the ASH-mediated locomotory circuit.

Each of the receptors that are essential for the serotonergic sensitization of aversive responses to dilute octanol operate at different levels within the ASH-mediated locomotory circuit: SER-5 in ASH sensory neurons, MOD-1 in the AIB interneurons that receive direct input from the ASHs, and SER-1 in RIA/ring motor neuron pathway that innervates head muscle (Fig. 7). Based on the wiring diagram of the *C. elegans* nervous system, we predict that SER-5 increases the responsiveness of the ASHs to initiate backward locomotion, MOD-1 modulates signaling to the command interneurons through the AIBs to inhibit forward locomotion, and SER-1 modulates signaling to head muscle (Fig. 7). For example, 5-HT stimulated responses in *ser-1* animals could be rescued with
a \textit{ser-1p::ser-1::gfp} transgene that is expressed in the RIAs and the RMD ring motor neurons that innervate head muscle (Xiao et al., 2006; Dernovici et al., 2007). Ablation of the RIAs or the knockdown of \textit{ser-1} expression in the RIAs has no effect on the rate of spontaneous reversal or octanol sensitivity off food, but abolishes 5-HT dependent increases in sensitivity to dilute octanol, suggesting that the sensitization of head muscle may be essential for stimulating aversive responses, especially when the ASH is less than maximally stimulated.
Figure 7. Tentative localization of 5-HT receptors in neurons involved in ASH-mediated aversive responses to dilute octanol. The ASH sensory neurons are necessary and sufficient for responses to dilute octanol and innervate multiple levels of the locomotory circuit, including the command interneurons that control forward and backward locomotion (White et al., 1986; Chao et al., 2004). Interestingly, based on the tentative localization of different ser-x::gfp transgenes and the neuron-specific rescue/knockdown studies described above, the 5-HT receptors modulating octanol sensitivity do not appear to be expressed directly in the command interneurons, but instead in neurons modulating their output. Direct outputs from the ASH are presented in red. Numbers are an estimate of synaptic connectives between neurons (White et al., 1986).
Food/serotonin activates SER-5 in the ASH sensory neurons.

Serotonin (5-HT) is one of the “food is at hand” signals in *C. elegans* and regulates most aspects of *C. elegans* behavior. Four *C. elegans* 5-HT receptors have been identified previously, three G-protein coupled receptors, SER-1, SER-4, and SER-7 that appear to signal through Gαq, Gαo, and Gαs, respectively, and a novel 5-HT gated chloride channel, MOD-1. In addition, we have identified a fifth putative 5-HT receptor, SER-5. Previously, the roles of these receptors in pharyngeal pumping (Hobson et al., 2003), egg-laying (Hobson et al., 2003; Carnell et al., 2005; Dempsey et al., 2005; Xiao et al., 2006), the enhanced slowing response (Ranganathan et al., 2000), and aversive learning (Zhang et al., 2005) have been characterized. In addition, 5-HT plays a role in modulating locomotory transitions associated with nutritional status (Fugiwara et al., 2002; Tsalik and Hobert, 2003; Gray et al., 2005). However, a thorough understanding of the anatomy and overall modulation of serotonergic signaling is still in its infancy and few studies have localized the 5-HT receptors involved in mediating individual behaviors.

The role of feeding status and 5-HT in the modulation of octanol avoidance has been demonstrated previously (Chao et al., 2004). The ASH sensory neurons are both necessary and sufficient for responses to dilute octanol and food/5-HT increases these responses. Similarly, nose touch, another ASH-mediated behavior, elicits 5-HT dependent Ca ++ transients in the ASHs, even in *unc-13* animals, strongly suggesting that 5-HT alters the sensitivity of the ASHs directly (Hilliard et al., 2005). Indeed, we have
identified a putative 5-HT receptor, SER-5, that functions directly in the ASHs. SER-5 is
most closely related to mammalian 5-HT₆ receptors. 5-HT₆ receptors are abundantly
expressed in olfactory tubercles, although no studies have examined their role in
mammalian nociception (Woolley et al., 2004; Mitchell and Neumaier, 2005). Since the
5-HT specificity of SER-5 has not been demonstrated through ligand-binding studies, it is
possible that an additional, as yet unidentified, 5-HT receptor is involved in the release of
another neuromodulator that then activates SER-5. We consider this a remote possibility,
given the close alignment of the new SER-5 sequence with other 5-HT receptors and the
identification of a second 5-HT stimulated behavior that is dependent on SER-5, i.e., 5-
HT dependent increases in egg-laying in ser-4;mod-1;ser-7 ser-1 null animals (Hapiak et
al., submitted). Attempts to express SER-5 in mammalian cells with the addition of
signal sequences or temperature shock, as we have described for other C. elegans
GPCRs, have been unsuccessful (Rex and Komuniecki, 2002; Hobson et al., 2003).
Indeed, the direct characterization of SER-5 may be problematic in the absence of a
nematode cell line. For example, the heterologous expression of many olfactory and
pheromone GPCRs that are expressed in sensory neurons is poor and may require
accessory proteins (Loconto et al., 2003).

Both the AIB and potentially the AIY interneurons are involved in the MOD-1-
dependent sensitization of ASH-mediated aversive responses.

The ASH sensory neurons innervate the AVA/AVB/AVD command interneurons
that likely function as a “bistable switch” to regulate forward/backward locomotion and
reversal (Chalfie et al., 1985; Zheng et al., 1999; Brockie et al., 2001; Tsalik and Hobert,
2003; Gray et al., 2005). In addition, the ASHs synapse on the AIA, AIB and RIA interneurons, but not on the motorneurons that directly innervate body wall muscle. 5-HT stimulated responses to dilute octanol can be rescued in mod-1 animals by expression of mod-1 transgenes in the AIBs or AIYs (npr-9p or ttx-3p). Similarly, the expression of odr-2(2b)p::mod-1 (AIBs/AIZs ) or ttx-3p::mod-1 transgenes rescued aversive learning in mod-1 animals (Zhang et al., 2005). The AIBs are innervated by the ASHs and are prominent postsynaptic partners of the AIAs. Since the expression of mod-1 in the AIBs alone rescued 5-HT dependent increases in octanol sensitivity, these results suggest that the AIBs play a key role in mediating the effects of MOD-1 signaling on octanol sensitivity (White et al., 1986).

The AIYs also appear to be involved in mod-1-mediated aversive behaviors, even though they are not directly innervated by the ASHs. In fact, many sensory neurons mediate locomotory behaviors through the AIYs (Ryu and Samuel, 2002; Tsalik and Hobert, 2003; Zariwala, 2003). For example, ttx-3 mutants, in which the AIYs fail to develop, do not modulate reversal frequency in response to ASE-mediated gustatory or AWA/AWC-mediated olfactory cues (Tsalik and Hobert, 2003). In contrast, in the present study, ttx-3 null animals exhibited wild-type aversive responses to dilute octanol in both the presence and absence of 5-HT. However, mod-1 encodes a 5-HT-gated chloride channel that would presumably inhibit AIY function. Therefore, as observed in the present study, aversive responses to dilute octanol on food would be predicted to be stimulated in both wild type animals without AIYs, as observed in ttx-3 animals or in animals in which the AIYs are inhibited by MOD-1, as in the ttx-3p::mod-1 rescued animals. It is worth noting that the expression of an inhibitory Cl\` channel has the
potential to silence neurons and could affect behavior, even if these neurons are not normally involved in the behavior. However, based on expression of mod-1 and neuron-specific rescue of mod-1 animals, these results suggest that both the AIBs and AIYs are involved in the MOD-1-dependent modulation of octanol sensitivity in the presence of food.

Both the AIBs and AIYs also modulate spontaneous reversal (Tsalik and Hobert, 2003; Gray et al., 2005). For example, AIB ablation prevents the stimulation of long reversals and omega turns when animals are removed from food, suggesting that the AIBs increase the probability of reversal during area restricted search (Gray et al., 2005). Similarly, ablation of the AIYs increases the frequency of spontaneous reversals both on and off food and roles have been proposed for the AIYs in modulating reversals associated with other behaviors (Mori and Oshima, 1997; Tsalik and Hobert, 2003; Gray et al., 2005). The AIBs and AIYs appear to act, at least in part, in parallel, based on the observation that their roles in the modulation of locomotory behavior appear to be additive (Gray et al., 2005). In the present study, spontaneous reversal was similarly stimulated in mod-1 and ttx-3 animals off food. In contrast, reversal frequency was inhibited in mod-1 animals and stimulated in ttx-3 animals on food. Together with the data on octanol sensitivity described above, these observations highlight the different, but overlapping, roles played by serotonergic signaling in the modulation of spontaneous reversals or ASH-mediated aversive responses.

In summary, our observations suggest that the serotonergic modulation of aversive responses involves sensitization at multiple levels within the ASH-mediated circuit that controls reversal/backward locomotion. In addition, they highlight the
advantages of the *C. elegans* model in dissecting the complex aminergic modulation of olfactory behaviors.
4.0 CHAPTER IV

The monoaminergic modulation of sensory-mediated aversive responses in Caenorhabditis elegans requires glutamatergic/peptidergic cotransmission


4.1. Results:

Both Gαq (EGL-30) and Gαs (GSA-1) signaling in the ASH sensory neurons increase aversive responses to dilute octanol

The ASH sensory neurons are necessary and sufficient for aversive responses to dilute octanol and the expression of the G-protein coupled receptor (GPCR), ser-5, in the ASHs is essential for food or 5-HT stimulation of the aversive response (Chao et al., 2004; Harris et al., 2009). For example, ser-5 null animals do not increase aversive responses in the presence of food or 5-HT, in contrast to wild type animals, and 5-HT stimulation can be restored by the expression of ser-5 in the ASHs (Fig. 8; Harris et al., 2009). Conversely, wild type animals overexpressing ser-5 exhibit more rapid responses off food (Fig. 8). To characterize the signaling pathways involved in the SER-5 mediated modulation of the ASHs, individual G-protein signaling mutants were examined for
octanol avoidance, including \( \text{lf} \) mutants for a PDE4 cAMP phosphodiesterase (\( pde-4 \), Charlie et al., 2006) and gain-of function mutants for \( \text{G} \alpha_{s} \) (\( gsa-1gf \), Schade et al., 2005), \( \text{G} \alpha_{q} \) (\( egl-30gf \), Hawasli et al., 2004) and adenylyl cyclase (\( acy-1gf \), Shade et al., 2005). Animals with increased \( \text{G} \alpha_{q} \) (\( egl-30gf \)) or \( \text{G} \alpha_{s} \) (\( gsa-1gf, pde-4 \)) signaling exhibited more rapid responses to dilute octanol off food than wild type animals that mimicked responses observed in the presence of food or 5-HT, suggesting that both signaling pathways might be involved in the food/5-HT sensitization of the ASHs (see diagram, Fig. 8).

To confirm that these G-protein signaling pathways were stimulating the ASHs directly, ASH-selective RNAi was used to specifically interfere with G-protein signaling (Esposito et al., 2007). This RNAi approach is reported to yield efficient, heritable, knockdown that does not spread significantly to other neurons, at least in the case of the ASHs (Esposito et al., 2007). We have confirmed these observations using the ASH-selective promoter, \( \text{sra-6p} \). For example, the ASH knockdown of \( \text{ser-5} \) or \( \text{octr-1} \) abolished the 5-HT stimulation and OA inhibition of aversive responses, respectively, but had no effect on responses off food, suggesting that the octanol-sensing machinery in the ASHs was intact (Fig. 9). Since the \( \text{sra-6} \) promoter also has limited expression in the ASI sensory neurons, an ASI-specific promoter (\( \text{gpa-4p} \)) was used as a control to knockdown many of the genes examined in the present study. As predicted, the ASI RNAi knockdown of \( \text{ser-5} \) or \( \text{octr-1} \) had no effect on aversive responses, confirming that our results were based on knockdown in the ASHs and also that spreading of the RNAi is limited (Fig. 9).

The ASH-selective RNAi knockdown of \( \text{G} \alpha_{q} \) (\( egl-30 \)) decreased aversive responses off food and the knockdown of either \( \text{G} \alpha_{q} \) or \( \text{G} \alpha_{s} \) (\( gsa-1 \)) abolished 5-HT
stimulation (Fig. 9). In contrast, the ASH RNAi knockdown of either \textit{pde-4}, a 5’ phosphodiesterase (Charlie et al., 2006) or \textit{kin-2}, a regulatory subunit of the cAMP dependent protein kinase (Lu et al., 1990), increased aversive responses off food to levels similar to those observed with 5-HT. Similarly the ASH RNAi knockdown of \textit{dgk-1}, a diacylglycerol (DAG) kinase also increased aversive responses off food to levels similar to those observed with 5-HT, presumably by increasing DAG levels downstream of ASH \( G_{\alpha_q} \) signaling (Miller et al., 1999; Nurrish et al, 1999). Together these results suggest that activating either \( G_{\alpha_s} \) or \( G_{\alpha_q} \) signaling sensitized the ASHs (Fig. 9).

To characterize the role of SER-5 in ASH sensitization, we attempted to increase aversive responses in \textit{ser-5} null animals by activating individual G-protein mediated pathways. Both \textit{egl-30gf ser-5} and \textit{egl-30gf} animals exhibited increased responses compared to \textit{ser-5} animals (Figs. 8 and 10). Similarly, the ASH RNAi knockdown of \textit{dgk-1} in either wild type or \textit{ser-5} animals also increased aversive responses both off food and in the presence of 5-HT (Figs. 9 and 10). Together, these results suggest that \( G_{\alpha_q} \) (EGL-30) functions downstream or in parallel to \textit{ser-5}; however, whether SER-5 couples directly to \( G_{\alpha_q} \) and stimulates neurotransmitter release or acts upstream to modulate neuronal excitability remains to be determined. In contrast, \textit{gsa-1gf ser-5}, \textit{ser-5:pde-4} or \textit{ser-5} animals with \textit{pde-4} knocked down in the ASH by RNAi did not exhibit the elevated aversive responses observed by activating \( G_{\alpha_s} \) signaling in the ASHs of wild type animals (Fig. 10). These results suggest that SER-5-dependent \( G_{\alpha_q} \) activation is essential for \( G_{\alpha_s} \)-stimulated increased responses off food.
Figure 8. Monoamine-mediated G-protein signaling modulates ASH-mediated aversive responses to dilute octanol. Wild-type and G-protein signaling mutants were examined for aversive responses to dilute (30%) octanol in the presence and absence of exogenous 5-HT, OA or 5-HT and OA (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. "*" P < 0.001, significantly different from wild-type animals under identical test conditions. Black off food; grey +OA; white +5-HT; hatched +5-HT+OA.
Figure 9. Monoamines modulate G-protein signaling in the ASH sensory neurons.

Wild-type animals expressing RNAi transgenes were assayed for aversive responses to dilute (30%) octanol in the presence or absence of exogenous 5-HT, OA or 5-HT and +OA (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals under identical test conditions. Black off food; grey +OA; white +5-HT; hatched +5-HT+OA. ASH selective promoter, sra-6p and ASI selective promoter, gpa-4p.
Figure 10. SER-5 signaling in ASH sensory neurons. Wild-type and mutant animals were examined for aversive responses to dilute (30%) octanol either in the presence or absence of exogenous 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P < 0.001, significantly different from wild-type animals under identical test conditions. ASH selective promoter, sra-6p.
Octopamine inhibits aversive responses by activating OCTR-1 and G\(\alpha_o\) in the ASHs

OA inhibits food or 5-HT stimulated aversive responses to dilute octanol through the OA receptor, OCTR-1, expressed in the ASHs (Wragg et al., 2007; Fig. 8). For example, 5-HT stimulated aversive responses were not inhibited by OA in octr-1 null animals or in wild type animals with octr-1 knocked down in the ASHs by RNAi (Figs. 8 and 9; Wragg et al., 2007). OA also inhibited the increased aversive responses observed off food in animals with elevated cAMP levels (gsa-1gf, acy-1gf, pde-4; Fig. 8).

Similarly, OA inhibited the elevated responses off food observed after the ASH RNAi knockdown of pde-4 (Fig. 9). In contrast, the ASH RNAi knockdown of G\(\alpha_o\) (goa-1) abolished the OA inhibition of 5-HT stimulation, but had no effect on responses off food or in the presence of 5-HT, suggesting that OA and OCTR-1 signal through G\(\alpha_o\) in the ASHs to inhibit 5-HT stimulation (Fig. 9). Presumably, the absence of elevated signaling off food after G\(\alpha_o\) knockdown reflects a paucity of endogenous OA and therefore, limited G\(\alpha_o\) activation.

OA also inhibited increased responses off food in G\(\alpha_q\) (egl-30gf) animals and this OA inhibition appeared to involve signaling in the ASHs, as the OA inhibition of egl-30gf animals was abolished by the ASH RNAi knockdown of octr-1 (Figs. 8 and 12). G\(\alpha_o\) may decrease signaling in the ASHs by inhibiting adenylyl cyclase. Alternatively, since G\(\alpha_o\) (GOA-1) inhibits G\(\alpha_q\) signaling, the ability of OCTR-1/ G\(\alpha_o\) signaling to abolish the G\(\alpha_q\) signaling responses is consistent with our finding that the increased off-food aversive response in mutants with an activated G\(\alpha_s\) pathway requires the SER-5 receptor and G\(\alpha_q\) (Hajdu-Cronin et al., 1999 and Miller et al., 1999; Figs 9 and 10).

EAT-16 encodes an inhibitory regulator of G-protein signaling (RGS) protein that
converts G\(_{q}\) from an active GTP-bound to an inactive GDP-bound form and \(l^f\) mutations in \textit{eat-16} animals were identified in a screen for suppressors of activated G\(_{s}\) (Hajdu-Cronin et al., 1999). Interestingly, OA did not inhibit the 5-HT stimulation of aversive responses in \textit{eat-16} null animals or in animals with \textit{eat-16} knocked down in the ASHs by RNAi (Figs. 8 and 9). The ASH RNAi knockdown of \textit{eat-16} had no effect on aversive responses off food or in the presence of 5-HT, as also observed after the ASH RNAi knockdown of G\(_{s}\) (Fig. 9). In contrast, OA did not inhibit the increased responses off food observed after the ASH RNAi knockdown of \textit{dgk-1} (Fig. 9).

These studies suggest that OA and OCTR-1 signal through G\(_{s}\) (GOA-1) in the ASHs and may inhibit the 5-HT stimulation of aversive responses by potentially turning off SER-5/G\(_{q}\) signaling, which our earlier data show is necessary for the G\(_{s}\) response. Interestingly, the OA-mediated inhibition of G\(_{q}\) and G\(_{s}\) driven ASH signaling appears to be sufficient to abolish food or 5-HT dependent increases in aversive responses.

**Peptides from the ASHs are required for the 5-HT stimulation of aversive responses**

As noted above, ASH G\(_{s}\) signaling was essential for the 5-HT stimulation of aversive responses. To determine if G\(_{s}\) signaling stimulated DCV/neuropeptide release, the potential role of neuropeptides in the aminergic modulation of ASH-mediated aversive responses was examined in \textit{egl-3} and \textit{egl-21} animals that lack a proprotein convertase and a carboxypeptidase, respectively, that are required for efficient neuropeptide processing (Fig.11). Both \textit{egl-3} and \textit{egl-21} animals exhibit severely deficient peptide profiles (Husson et al., 2006, 2007). Indeed, both \textit{egl-3} and \textit{egl-21} animals failed to increase aversive responses in the presence of 5-HT, suggesting that
peptides are essential for 5-HT stimulation. In contrast, egl-3 and egl-21 animals off food exhibited wild type responses to dilute octanol, suggesting that their octanol-sensing machinery was intact. Similar phenotypes were observed after the ASH RNAi knockdown of egl-3, confirming a role for egl-3 in the ASHs (Fig. 12).

The ASHs express at least three neuropeptide genes (nlp-3, nlp-15 and flp-21) that encode at least nine predicted peptides (Nathoo et al., 2001). In the presence of exogenous 5-HT, aversive responses to dilute octanol in nlp-15 and flp-21 null animals were identical to those of wild type animals (data not shown). In contrast, aversive responses were not stimulated by food or 5-HT in nlp-3 null animals (Figs. 11-13). Conversely, the overexpression of nlp-3 in wild type animals from either the native nlp-3 or ASH-selective promoters increased aversive responses off food (Fig. 11). nlp-3 is expressed in many sensory neurons, including the ADFs, ASEs, ASHs, ASJs, and AWBs (Nathoo et al., 2001). However, 5-HT stimulation was fully rescued in nlp-3 animals by the ASH-selective expression of nlp-3 and the ASH-selective RNAi knockdown of nlp-3 abolished food or 5-HT stimulation to levels observed in the nlp-3 null animals (Figs. 11-13). Together, these results suggest that the release of peptides encoded by nlp-3 from the ASHs is sufficient to stimulate aversive responses to dilute octanol.
Figure 11. Peptides encoded by nlp-3 are essential for the 5-HT dependent stimulation of aversive responses to dilute octanol. Wild-type, mutant and transgenic animals were examined for aversive responses to dilute (30%) octanol in the absence (top): or presence (Bottom): of exogenous 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals under identical test conditions. ASH selective promoter, sra-6p.
Figure 12. The release of peptides encoded by nlp-3 is essential for Gαs stimulation of aversive responses mediated by from the ASH sensory neurons. Animals were examined for aversive responses to dilute octanol (30%) in the absence of food or exogenous 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals under identical test conditions. Black off food; White +5-HT; Grey +OA. ASH selective promoter, sra-6p.
Figure 13. The RNAi knockdown of *nlp-3* in the ASHs abolishes the food 
stimulation of aversive responses to dilute octanol. Aversive responses to dilute octanol were examined in wild type animals in the presence of *E. coli* (OP50) after the promoter selective RNAi knockdown of *nlp-3* (ASHs, *sra-6p::nlp-3*; NSMs, *ceph-2p::nlp-3*, ADFs, *srh-142p::nlp-3*; AWBs, *str-1p::nlp-3*). Data are presented as a mean ± SE and analyzed by two-tailed Student’s *t* test. “*” *P* < 0.001, significantly different from wild-type animals under identical test conditions. Grey, + food.
\( \text{G}_{\alpha_s} \) (GSA-1) signaling in the ASHs appears to stimulate the release of peptides encoded by \( nlp-3 \)

The ASH RNAi knockdown of \( egl-3 \), in \( gsa-1gf \) animals abolished the increased aversive responses observed in these \( gf \) mutants off food, suggesting the \( \text{G}_{\alpha_s} \) signaling was stimulating peptide release (Fig. 12). Therefore, to identify the G-protein signaling pathways specifically modulating the release of \( nlp-3 \) encoded peptides from the ASHs, additional mutants were created. In agreement with the \( egl-3 \) data, \( gsa-1gf; nlp-3 \) and \( pde-4; nlp-3 \) animals did not exhibit the elevated responses off food observed in \( gsa-1gf \) or \( pde-4 \) animals (Fig. 12). Similarly, the ASH RNAi knockdown of \( pde-4 \) in \( nlp-3 \) null animals also failed to increase responses off food, in contrast to the ASH RNAi knockdown of \( pde-4 \) in wild type animals (Figs. 9 and 12). In contrast, both \( egl-3\text{gf} \) and \( egl-3\text{gf}; nlp-3 \) animals exhibited elevated responses off food (Figs. 8 and 12). Similarly, the ASH RNAi knockdown of \( dgk-1 \) in either wild-type, \( egl-3 \) or \( nlp-3 \) animals also stimulated responses off food, suggesting the \( nlp-3 \) was not downstream of \( \text{G}_{\alpha_q} \) and that \( \text{G}_{\alpha_q} \)-stimulated increases in glutamate alone might be sufficient to increase ASH-mediated signaling (Figs. 9 and 12).

Together, these results suggest that ASH-mediated aversive responses to dilute octanol can be increased by either 1) activating \( \text{G}_{\alpha_q} \) signaling by the overexpression or activation of \( \text{SER}-5/\text{EGL}-30 \) or the inhibition of \( \text{DGK}-1 \) or 2) activating \( \text{G}_{\alpha_s} \) signaling and peptide release through the activation \( \text{GSA}-1/\text{ACY}-1 \), the inhibition of \( \text{PDE}-4 \) or the overexpression of peptides encoded by \( nlp-3 \).
Serotonin stimulates aversive responses when glutamatergic signaling in the ASHs is compromised through a pathway requiring peptides encoded by nlp-3.

To confirm the stimulatory role of nlp-3 encoded peptides on ASH-mediated aversive responses, eat-4 null animals that lack a vesicular glutamate transporter essential for glutamatergic transmission were examined (Lee et al., 1999). Not surprisingly, eat-4 animals or wild type animals with eat-4 knocked down by RNAi in the glutamatergic ASHs responded very poorly (> 18 and 16 sec, respectively), if at all, to dilute octanol, since these assays were terminated after 20 sec to minimize complications introduced by spontaneous reversal (Fig. 14). However, aversive responses in eat-4 animals or animals with eat-4 knocked down in the ASHs by RNAi were still dramatically stimulated by 5-HT (Fig. 14). In contrast, aversive responses in eat-4;egl-3, eat-4;nlp-3 animals, or eat-4 null animals with nlp-3 knocked down in the ASHs by RNAi were not stimulated by 5-HT, suggesting that 5-HT was stimulating aversive responses by stimulating the release of nlp-3 encoded peptides in the eat-4 animals (Fig. 14). This apparent 5-HT stimulation of peptide release was unanticipated, given that SER-5 appears to signal through a pathway requiring Gαq and peptide release appears to be dependent on Gαs (see Figs. 8 and 9). Interestingly, the knockdown of either Gαs, Gαq or UNC-13 in eat-4 animals abolished 5-HT stimulation (Fig. 14).

Together, these results demonstrate that 1) in the absence of the ASH EAT-4 vesicular glutamate transporter, animals respond very poorly to dilute octanol, supporting a role for EAT-4 as the major ASH glutamate transporter, 2) aversive responses in eat-4 animals are still stimulated by 5-HT, while aversive responses in eat-4;nlp-3 or eat-4 animals with nlp-3 knocked down in the ASHs are not, further supporting a role for nlp-3.
in 5-HT stimulation and 3) in the absence of significant glutamatergic signaling both
ASH \( \Gamma \alpha_s \) and \( \Gamma \alpha_q \) signaling are essential for 5-HT stimulation.
Figure 14. 5-HT stimulates ASH-mediated aversive responses in eat-4 null animals.

Wild-type and mutant animals were examined for aversive responses to dilute octanol (30%) in the presence or absence of exogenous 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from animals incubated in the absence of 5-HT. “**” Significantly different from eat-4 null animals incubated under identical conditions. Black off food; White +5-HT. ASH selective promoter, sra-6p.
4.20 Discussion:

Behavioral state in *C. elegans* is dependent on food availability and is defined by the selective release of both monoamines and peptides. For example, 5-HT/OA ratios define nutritional status and antagonistically modulate most key behaviors (Horvitz, 1982; Alkema et al., 2005; Wragg et al., 2007). The present studies demonstrate that 5-HT and OA also antagonistically modulate aversive behaviors mediated by the ASHs and that release of *nlp-3* peptides from the ASHs is essential for the 5-HT stimulation of aversive responses. Both G*\(_\alpha_q\)* and G*\(_\alpha_s\)* signaling in the ASHs are essential for 5-HT sensitization, with G*\(_\alpha_s\)* signaling specifically stimulating neuropeptide release (see Fig. 15). The role of G-protein signaling in modulating aversive responses in the ASHs is consistent with that predicted for neurotransmitter release from *C. elegans* motoneurons (Perez-Mansilla and Nurrish, 2009). For example, the G*\(_\alpha_s\)* pathway also exerts its effects on locomotion in a G*\(_\alpha_q\)* pathway-dependent manner (Reynolds et al., 2005). However, whether these G-protein signaling cascades directly modulate glutamate and/or neuropeptide release remains to be determined. For example, ASH signaling requires the activity of an OSM-9/OCR-2 TRPV ion channel in the sensory cilia and in other systems G*\(_\alpha_q\)* signaling is involved in TRPV activation (Colbert et al., 1997; Montel, 2005; Bergamsco and Bazzicalupo, 2006, for review). Similarly, gap junctions are also modulated by monoaminergic signaling (Rorig and Sutor, 1996; Bloomfield and Volgyi, 2009; Kothmann et al., 2009). The ASHs offer advantages over motoneurons for studies of G-protein signaling and behavioral plasticity. For example, ASH postsynaptic partners are neurons, not muscles, Ca\(^{++}\) dynamics are modulated directly by sensory cues (Hilliard et al., 2005), modulatory peptides and monoamine/peptide receptors have been identified,
and the ASHs are amenable to RNAi permitting the selective manipulation of ASH signaling directly. In contrast, we have observed off target effects using this RNAi approach in cholinergic motorneurons, probably, in part, because of the large number of motorneurons expressing the dsRNAs.
Figure 15. Model of G-protein modulation of aversive responses mediated by the ASH sensory neurons. Green and red represent stimulation and/or inhibition, respectively, of aversive responses to dilute octanol.
**G-protein signaling in the ASHs modulates aversive responses.**

Activating either $\text{G}_\alpha_q$ or $\text{G}_\alpha_s$ signaling in the ASHs increases aversive responses. In contrast, inhibition of either $\text{G}_\alpha_q$ or $\text{G}_\alpha_s$ signaling in the ASHs abolishes 5-HT stimulation. These results suggest that 5-HT stimulation requires both $\text{G}_\alpha_q$ and $\text{G}_\alpha_s$ signaling, but that the activation of either pathway is sufficient to increase aversive responses, as long as basal levels of signaling in the other pathway are maintained. The receptor activating $\text{G}_\alpha_s$ in the ASHs has not been identified, but does not appear to be SER-5. For example, $\text{gsa-1gf ser-5}$ or $\text{ser-5;pde-4}$ mutants or $\text{ser-5}$ animals with $\text{pde-4}$ knocked-down in the ASHs are not stimulated by 5-HT and do not exhibit the elevated responses off food observed in $\text{gsa-1gf}$ or $\text{pde-4}$ animals, suggesting that $\text{G}_\alpha_s$ does not function downstream of SER-5. In contrast, the ASH RNAi knockdown of $\text{ser-5}$ has no effect on the increased aversive responses off food associated with increased $\text{G}_\alpha_q$ signaling, suggesting that $\text{G}_\alpha_q$ functions downstream of SER-5; whether SER-5 couples directly to $\text{G}_\alpha_q$ remains to be determined.

OA inhibits the food/5-HT stimulation of aversive responses through OCTR-1 (F14D12.6) expressed in the ASHs (Wragg et al., 2007). OA also inhibits increased responses off food associated with increased $\text{G}_\alpha_s$ signaling and the ASH RNAi knockdown of either $\text{octr-1}$ or $\text{G}_\alpha_o$ abolishes the OA inhibition of food/5-HT dependent stimulation, suggesting that OCTR-1 activates $\text{G}_\alpha_o$. OA and $\text{G}_\alpha_o$ may inhibit ASH signaling by negatively regulating adenylyl cyclase and/or inhibiting $\text{G}_\alpha_q$, perhaps through the RGS protein, EAT-16, as OA did not inhibit the 5-HT stimulation of aversive responses in $\text{eat-16}$ null animals or animals with $\text{eat-16}$ knocked down in the ASHs. Clearly, $\text{G}_\alpha_o$ inhibits $\text{G}_\alpha_q$ signaling in *C. elegans*, but the specifics of $\text{G}_\alpha_o$ inhibition are
unclear (Hadju-Cronin et al., 1999, Miller et al. 1999). Both *dgk-1* (directly) and *eat-16* (indirectly) modulate DAG levels and *dgk-1* and *eat-16* null mutants were both identified in screens for suppressors of activated G<sub>αo</sub> (Hadju-Cronin et al., 1999; Miller et al. 1999; Nurrish et al., 1999)

ASH signaling can be operationally divided into three overlapping circuits that 1) stimulate backward locomotion via AVA/AVD command interneurons, 2) inhibit forward locomotion via AVB command interneurons and a second pathway innervating the RIMs via the AIA/AIB interneurons and 3) sensitize head muscle through the RIAs (Harris et al., 2009). A different 5-HT receptor is essential in each branch for food or 5-HT stimulation; SER-5 in the ASHs, MOD-1 in the AIBs and SER-1 in the RIAs (Harris et al., 2009). Clearly, the release of *nlp-3* encoded peptides are also essential for 5-HT stimulation and will be critical for understanding the role of peptidergic signaling in monoaminergic modulation. For example, 5-HT and/or NLP-3 could modulate downstream postsynaptic glutamate receptors, neuronal excitability, gap junctions and/or pre-synaptic neurotransmitter release.

**G<sub>α</sub>s signaling stimulates the release of peptides encoded by *nlp-3* from the ASHs.**

Neurotransmitters are secreted from synaptic vesicles (SVs) that contain classical neurotransmitters, such as glutamate, and dense core vesicles (DCVs) that contain neuropeptides that activate GPCRs to modulate the sensitivity of both pre and post-synaptic neurons. Although factors regulating exocytosis from SVs and DCVs are similar, the DAG-binding proteins, UNC-13 (MUNC-13-1) and UNC-31 (CAPS) play
selective roles in exocytosis from SVs and DCVs, respectively, with UNC-31 involved in the docking of fusion-competent DCVs (Gracheva et al., 2007; Hammarlund et al., 2007; Speese et al., 2007; Zhou et al., 2007). $G_{\alpha_s}$ signaling and PKA activation enhance exocytosis from DCVs. For example, in *C. elegans*, PKA activation rescues the uncoordinated phenotype of *unc-31* mutants, bypasses the requirement for UNC-31 in the docking of DCVs, and directly stimulates DCV exocytosis (Charlie et al., 2006; Zhou et al., 2007).

Our data suggests that activating $G_{\alpha_s}$ signaling in the ASHs stimulates peptide release that, in turn, increases ASH-mediated aversive responses. For example, 1) the more rapid responses off food associated with increased $G_{\alpha_s}$ signaling are absent in *gsa-1gf;nlp-3* or *pde-4;nlp-3* animals, 2) the ASH RNAi knockdown of *pde-4* fails to increase responses off food in *nlp-3* animals, and 3) the ASH RNAi knockdown of *nlp-3* in *pde-4* animals abolishes increased responses observed in *pde-4* animals off food. These data suggest that $G_{\alpha_s}$-dependent sensitization of the ASHs requires the release of peptides encoded by *nlp-3*. In contrast, the increased aversive responses associated with increased $G_{\alpha_q}$ signaling off food did not require *nlp-3*, suggesting that DCV exocytosis in the ASHs may not be essential for $G_{\alpha_q}$-mediated stimulation. For example, the ASH RNAi knockdown of *nlp-3* had no effect on the increased responses off food in *egl-30gf* animals and the ASH RNAi knockdown of *dgk-1* increased responses off food in both wild type and *nlp-3* null animals. Presumably, $G_{\alpha_q}$ signaling increases the UNC-13-dependent release of glutamate from SVs.
Together, these studies demonstrate that 5-HT and OA antagonistically modulate the ASH sensory neurons and that the Goα5-dependent release of nlp-3 encoded peptides from the ASH is essential for 5-HT stimulation.
5.0 CHAPTER V

Dissecting the food-signal from the serotonergic neurons sensitizing aversive responses mediated by *C. elegans* sensory neurons

Harris G. P, Korchnak A, Komuniecki R. W

5.1 Results:

The release of 5-HT from the NSMs, but not the ADFs, is required for the food-stimulation of aversive responses mediated by the ASH sensory neurons

Food or 5-HT dramatically stimulate ASH-mediated aversive responses to dilute octanol and *tph-1* null animals that lack tryptophan hydroxylase, the rate-limiting enzyme for 5-HT biosynthesis, fail to modulate aversive responses on food. *C. elegans* contains nine serotonergic neurons, four pairs of bilaterally symmetrical ADFs, NSMs, HSNs, and AIMs and the RIH (Horvitz et al., 1982; Sze et al., 2000). The ADFs, NSMs and HSNs express *tph-1* (Sze et al., 2000). In contrast, the AIMs and RIH do not appear to synthesize 5-HT de novo and instead accumulate 5-HT released from the biosynthetic neurons through a 5-HT transporter, MOD-5 (Sze et al., 2000). Importantly, 5-HT from the NSMs and ADFs may exhibit unique modulatory roles, as ADF 5-HT is required for aversive responses to pathogenic bacteria and NSM 5-HT for enhanced slowing on food
(Sawin et al., 2000; Ranganathan et al., 2004; Zhang et al., 2005). Therefore, to identify the source of the 5-HT mediating the food-dependent modulation of ASH-mediated aversive responses, the roles of the NSMs and ADFs in food stimulation was examined using neuron-specific rescue of *tph-1* null mutants and *tph-1* RNAi knockdown in wild-type animals. This RNAi approach has been validated previously in the ASH sensory neurons and appears to also function efficiently in the NSMs and ADFs with no apparent spreading (Esposito et al., 2007; Harris et al., 2009; Harris et al., 2010).

The food-dependent stimulation of aversive responses in *tph-1* null animals was rescued by the expression of *tph-1* in the NSMs, but not the ADFs (Fig. 16). Similarly, food-dependent stimulation was abolished by the RNAi knockdown of *tph-1* in the NSMs, but not the ADFs, of wild-type animals (Fig. 16). To further confirm a role for the NSMs in food-sensitization, RNAi was also used to selectively knockdown *unc-86* in the NSMs and *osm-9* in the ADFs, based on the observation that UNC-86 (POU domain containing transcription factor) and OSM-9 (TRP channel subunit) were essential for 5-HT synthesis in the NSMs and ADFs, respectively (Sze et al., 2002; Zhang et al., 2004; Sze et al., 2007; Fig. 16). As predicted, the NSM knockdown of *unc-86* abolished food-sensitization, but the ADF knockdown of *osm-9* had no effect on aversive responses (Fig. 16). Together, these results suggest that 5-HT signaling from the NSMs, but not the ADFs are required for the food stimulation of all aspects of reversal behavior associated with ASH mediated aversive responses.
Figure 16. The release of 5-HT from the NSMs, but not the ADFs, is required for food-stimulation of aversive responses mediated by the ASH sensory neurons.

Wild-type, mutant and transgenic animals were examined for aversive responses to dilute octanol (30%) in the presence of food, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals incubated in the presence of food.
5-HT from the ADFs may inhibit ASH-mediated aversive responses

To further define the potential roles of 5-HT released from the NSMs and ADFs, *mod-5* that encodes the only 5-HT transporter in *C. elegans* was selectively knocked down in the NSMs or ADFs, using neuron-selective RNAi (Horvitz et al., 2001). This *mod-5* knockdown would be predicted to prevent 5-HT reuptake and increase 5-HT levels at the site(s) of NSM or ADF 5-HT release, although effects on global 5-HT levels cannot be ruled out. Aversive responses were stimulated both off and on food in *mod-5* null animals and in animals with *mod-5* knocked down in the NSMs by RNAi, presumably because NSM 5-HT accumulation was increased (Fig. 17). Similarly, NSM *tph-1* overexpression also produced a hypersensitive response on food, further supporting a stimulatory role for NSM 5-HT on food (Fig. 16). Not surprisingly, *tph-1* over-expression had no effect on basal aversive responses off food, presumably because 5-HT release was not stimulated under these conditions. In contrast, the RNAi knockdown of *mod-5* in the ADFs abolished food stimulation, suggesting that 5-HT released from the ADFs inhibits ASH mediated aversive responses (Fig. 17). Indeed, the overexpression of *tph-1* in the ADFs of wild-type animals also abolished food-stimulation (Fig. 16). Together, this data confirms a stimulatory role for 5-HT released from the NSMs and suggests that 5-HT released from the ADF may inhibit ASH-mediated aversive responses.
**Figure 17.** *mod-5* knockdown in the NSMs and ADFs increases and decreases ASH mediated aversive responses, respectively. Wild-type and RNAi knockdown animals were examined for aversive responses to dilute octanol (30%) in the presence or absence of food, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s *t* test. “*” *P* < 0.001, significantly different from wild-type animals incubated in the presence or absence of food.
INS-1 signaling from the NSMs and ADFs inhibits 5-HT release and antagonistically modulates ASH-mediated aversive responses.

The insulin-like peptide, INS-1, is expressed in many neurons, including the NSMs and ADFs, and ins-1 null animals exhibited more rapid aversive responses than wild-type animals off food, suggesting that INS-1 inhibited food-sensitization (Pierce et al., 2001). To determine if either NSM or ADF INS-1 was involved, the expression ins-1 was selectively knocked down using RNAi. NSM ins-1 RNAi knockdown in wild-type animals stimulated aversive responses both on and off food (Fig. 18). In contrast, the overexpression of ins-1 in the NSMs of wild-type animals abolished food-dependent increases in aversive responses (Fig. 18). Together, these results suggest that NSM INS-1 inhibits food stimulation, perhaps by inhibiting the synthesis/release of NSM 5-HT. Indeed, animals with both tph-1 and ins-1 knocked down in the NSMs did not exhibit the more rapid aversive responses off food observed after the NSM knockdown of ins-1 alone (Fig. 18). In contrast, the ADF RNAi knockdown of ins-1 abolished food stimulation, suggesting that the ADF ins-1 RNAi might be stimulating ADF 5-HT release and inhibiting aversive responses, in agreement with observations described above on the ADF knockdown of mod-5 or overexpression of tph-1 (Fig. 18). Indeed, animals with both ins-1 and tph-1 knocked down in the ADFs exhibited wild-type aversive responses that were stimulated by food. Interestingly, given that insulin activates IGF-1 signaling in mammals, daf-2 that encodes the insulin/IGF-1 receptor was knocked down in the NSMs using RNAi. NSM daf-2::RNAi had no effect on NSM INS-1 phenotypes, suggesting either that INS-1 did not signal through DAF-2 at least in the NSMs or that NSM INS-1 did not function cell autonomously and instead activating additional neurons.
in the circuit (data not shown). Importantly, DAF-2 independent INS-1 signaling has been demonstrated in the modulation of other key behaviors, although the mechanism and receptors remain to be identified (Gruninger et al., 2006; Chalasani et al., 2010).

Together, these results suggest that 1) the release of 5-HT from the NSMs stimulates and ADFs inhibits ASH-mediated aversive responses and 2) INS-1 released from these neurons may function cell autonomously to inhibit 5-HT release.
Figure 18. INS-1 signaling from the NSMs and ADFs inhibits the antagonistic 5-HT effects of ASH-mediated aversive responses. Wild-type, mutant, and transgenic animals were examined for aversive responses to dilute octanol (30%) in the presence or absence of food, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals incubated in the presence or absence of food.
The NSM expression of the vesicular glutamate transporter, EAT-4, is also essential for food-dependent sensitization of ASH-mediated aversive responses.

Many serotonergic neurons in the mammalian CNS exhibit glutamatergic/peptidergic co-transmission and the NSMs express EAT-4, the major vesicular glutamate transporter in *C. elegans*, suggesting that the NSMs may also be glutamatergic (Fig.19; Lee et al., 1999). NSM RNAi knockdown of *eat-4* has no effect on basal aversive responses off food or on increased responses on 5-HT, but completely abolishes increased aversive responses on food (Fig. 19). Conversely, the overexpression of *eat-4* in the NSMs of wild-type animals significantly increases aversive responses on food, but has no effect off food (Fig.19). Together, this data suggests that 1) *eat-4* is essential for the food-stimulation of ASH-mediated aversive responses. However, whether *eat-4* is required for NSM glutamate release or is involved in the increased loading of 5-HT into synaptic vesicles, as has recently been proposed for mammalian serotonergic neurons, remains to be determined (Amilhon et al., 2010).
Figure 19. The NSM expression of EAT-4 is essential for food-dependent sensitization of ASH-mediated aversive responses. Wild-type, RNAi knockdown and overexpressor animals were examined for aversive responses to dilute octanol (30%) in the presence or absence of food or 5-HT (4 mM), as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P < 0.001, significantly different from wild-type animals incubated in the presence or absence of food or 5-HT.
Distinct 5-HT receptors are involved in the NSM stimulation and ADF inhibition of aversive responses

As noted above, 5-HT signaling from the NSMs is essential for food-dependent increases in aversive responses, while 5-HT from the ADFs appears to inhibit aversive responses on food, suggesting that ADF signaling may be inhibited during the food-sensitization of aversive responses. Indeed, the food-dependent inhibition of ADF 5-HT has been observed previously in food dependent modulation of responses to hyperoxia (Chang et al., 2006). To identify the 5-HT receptors required for NSM 5-HT stimulation and ADF 5-HT inhibition on food, aversive responses were examined in animals with null alleles for individual 5-HT receptors after the selective RNAi knockdown of mod-5 or ins-1 in either the NSMs or ADFs, on the hypothesis that the knockdown of either ins-1 or mod-5 should selectively increase neuron-specific serotonergic signaling. The NSM knockdown of either ins-1 or mod-5 dramatically increased aversive responses both on and off food in wild type, ser-7, ser-4 and mod-1 animals, but had no effect on aversive responses in ser-1 or ser-5 animals, suggesting that both SER-5 and SER-1 were activated by the increased 5-HT from the NSMs (Fig. 20). This was somewhat surprising given that mod-1 is also essential for food and 5-HT dependent increases in octanol avoidance in wild-type animals (Harris et al., 2009, 2010). In contrast, the ADF knockdown of either ins-1 or mod-5 inhibited the food-sensitization of aversive responses in wild-type, ser-4, ser-5, ser-7 and mod-1 null animals, but had no effect on ser-1 null animals, suggesting that SER-1 was activated by the increased 5-HT released from the ADFs to inhibit food sensitization (Fig. 20). This result was surprising, as previous work had demonstrated and that the expression of ser-1 in the RIAs, major downstream
synaptic partners of the ADFs, was essential for food or 5-HT sensitization. These studies are continuing to identify the site of SER-1 action in these experiments and the source of 5-HT involved in MOD-1 stimulation.
Figure 20. Distinct 5-HT receptors are involved in NSM dependent stimulation and ADF dependent inhibition of aversive responses. Wild-type and 5-HT receptor null mutants expressing cell specific/selective mod-5 or ins-1 RNAi transgenes (Top: NSMp::RNAi Bottom: ADFp::RNAi) were examined for aversive responses to dilute octanol (30%) in the presence or absence of food, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from animals incubated in the presence or absence of food.
NSM 5-HT also modulates post-initiation responses regardless of the intensity of the initiating stimulus through multiple 5-HT receptors

In addition to modulating the initiation of reversal (time to reversal after exposure to stimulus), food also modulates the length of reversal (headswings/reversal), as well as directional decisions after the reversal is complete, i.e., food not only dramatically stimulates the time taken to reverse, but also modulates behavior once reversal is initiated. For example, on food, reversals are short (headswings/reversal) and after reversal is complete most animals continue forward along their previous path (<45° from initial trajectory). In contrast, off food, animals backup more extensively and turn significantly away from their previous trajectory (>45° from initial trajectory) (Fig. 21). These post-initiation phenotypes are independent of the intensity of the initiating stimulus, i.e., even though animals initiate reversal much more rapidly to 100% than 30% octanol, food has identical effects on post-initiation responses (Fig 21, data not shown). Exogenous 5-HT mimics food in these post-initiation assays and modulates the length and directionality of reversal, as post-initiation responses in tph-1 null animals on food are similar to those of animals off food (Fig. 22). To identify the source of 5-HT responsible for food dependent suppression of turns and reversals, animals expressing a tph-1 RNAi knockdown in the NSMs and ADFs were examined on food (Fig. 23). NSM tph-1 RNAi but not ADF tph-1 RNAi animals were defective for food dependent suppression of turns and reversal duration, suggesting 5-HT from the NSMs, but not the ADFs is required for the food stimulation of post-initiation responses. Interestingly, NSM or ADF knockdown of egl-3 did not effect food dependent suppression of turns and reversals on food, suggesting peptides processed by egl-3 at least in the NSMs or ADFs
are not required for postinitiation responses (data not shown). The three 5-HT receptors that are essential for the food sensitization of aversive responses to 30% octanol, SER-1, SER-5 and MOD-1 are also involved in the food sensitization of post-initiation responses based on ser-1, ser-5 and mod-1 null animals each failing to suppress turns and reversal duration on food in response to dilute octanol compared to wild-type animals (Fig. 22, 23).

Together these results demonstrate that NSM 5-HT not only sensitizes the ASHs to octanol avoidance, but also markedly alters post initiation behaviors, regardless of the intensity of the initiating stimulus.
Figure 21. Food and 5-HT modulates post-initiation behavior in response to dilute octanol. (A). Diagram representing post-initiation behavior measured in response to dilute octanol (B). Wild-type animals were examined for postinitiation responses to dilute octanol in the presence or absence of Top: food or Bottom: 5HT, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from animals incubated in the presence or absence of food or 5-HT.
Figure 22. Food dependent suppression of reversal duration and turns requires SER-1, SER-5 and MOD-1. Wild-type and mutant animals were examined for post-initiation responses to dilute octanol in the presence or absence of food, as described in
Methods. (A) Turning angle behavior (B) Number of head swings per reversal. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P< 0.001, significantly different from wild-type animals incubated in the presence or absence of food.
Figure 23. Food dependent modulation of post-initiation responses requires NSM but not ADF 5-HT. Wild-type and cell selective RNAi animals were examined for post-initiation responses to dilute octanol in the presence or absence of food. (A) Turning
angle behavior (B) Number of head swings per reversal, as described in Methods. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. “*” P < 0.001, significantly different from wild-type animals incubated in the presence or absence of food.
5.2 Discussion:

Food and 5-HT translate nutritional status into the modulation of most behaviors, including key locomotory transitions associated with food availability. However, little is known about the roles of the individual serotonergic neurons in modulating these processes. In the present study, we have dissected the food signal that modulates ASH-mediated signaling and demonstrated that 5-HT released from the serotonergic NSMs and ADFs have antagonistic effects on aversive behavior, with NSM 5-HT stimulating and ADF 5-HT inhibiting acute ASH-mediated aversive responses.

The NSMs are secretory neurons whose cell bodies are located within the pharynx region and whose free axonal endings extend into the pseudocoelom (Albertson and Thomson, 1976). The NSMs have numerous varicosities with no apparent synaptic specializations and contain numerous lightly and darkly staining membrane-bound vesicles that are assumed to secrete 5-HT and a variety of neuropeptides directly into the pseudocoelastic fluid. This anatomical arrangement suggests a neuroendocrine role, with the NSMs most probably acting in an endocrine fashion to globally modulate signaling through volume transmission (Albertson and Thomson, 1976; Sze et al., 2000, 2002). Interestingly, some mammalian monoaminergic neurons also contain no obvious synaptic specializations and are proposed to be involved in humoral or diffuse transmitter release (Trudeau et al., 2004; Descarrées et al., 1975). However, although the NSMs are pharyngeal neurons and 5-HT dramatically stimulates pharyngeal pumping, ablation of the NSMs has no obvious effect on pumping or feeding, so the primary function of the NSMs remains to be determined. Indeed, the NSMs are involved in multiple behaviors, including the enhanced slowing of starved animals on food and dispersal off food (Avery
and Thomas, 1976; Avery et al., 1993, 1997; Sawin et al., 2000; Lipton et al., 2004; Wakabayashi et al., 2004). The ADF sensory neurons detect external environmental signals and thereby contribute to the perception of the environment (White et al., 1986; Bargmann and Horvitz, 1991). The ADFs are innervated by the ASHs directly and synapse onto a number interneurons involved in the modulation of locomotory state, including the RIA interneurons previously identified in the serotonergic stimulation of ASH-mediated aversive responses (Harris et al., 2009). The ADFs have also been implicated in 5-HT dependent increases in aversive learning, dispersal off food, increased forward movement, chemotaxis, therotaxis, entry into dauer, stress responses and act as a convergence point for the regulation of hyperoxia avoidance (Horvitz et al., 1982; Bargmann and Horvitz, 1991; Janssen et al., 1999; Wakabayashi et al., 2004; Zhang et al., 2005; Wakabayashi et al., 2005; Chang et al., 2006; Hukema et al., 2006; Dernovici et al., 2007; Zubenco et al., 2008).

Food and 5-HT increase ASH-mediated aversive responses through at least three different 5-HT receptors, operating at different levels within the ASH-mediated locomotory circuit (Chao et al., 2004; Hilliard et al., 2005; Harris et al., 2009, 2010). Interestingly, 5-HT from the NSMs and ADFs appears to interact with different subsets of these 5-HT receptors in the modulation of aversive responses and it appears that food-dependent serotonergic signaling is characterized by precisely modulated changes in local 5-HT levels, probably involving a mixture of both synaptic and extra-synaptic 5-HT receptors (Fig. 24). In fact, C. elegans 5-HT receptors are expressed on many neurons that are not directly innervated by serotonergic neurons, suggesting that much of the
serotonergic signaling may be humoral and extra-synaptic (Ranganathan et al., 2000; Xiao et al., 2006; Dernovici et al., 2007).

Figure 24: Model representing serotonergic and peptidergic modulation of aversive responses through NSM and ADF signaling. Green and red represent stimulation and/or inhibition, respectively, of aversive responses to dilute octanol on food.
**Serotonergic signaling from the NSMs and ADFs, produces antagonistic effects on aversive behaviors**

The present study has demonstrated that NSM and ADF serotonergic signaling act antagonistically, with NSM 5-HT stimulating and ADF 5-HT inhibiting ASH-mediated aversive responses to dilute octanol. Interactions between NSM and ADF signaling are complex, with neuron-specific and cooperative interactions also described for different behaviors, although few studies have thoroughly examined the roles of these neurons individually in the modulation of specific behaviors. For example, both NSMs and ADFs are required for dispersal after extended periods off food (Wakabayashi et al., 2004). In contrast, the ADFs also appear to exert NSM-independent effects. For example, the ADFs are required for DAF-dependent stress responses, as well as hyperoxia responses on food, aversive learning to pathogenic bacteria, chemotaxis to water soluble compounds, thermotaxis, entry into dauer stage and regulation of foraging behavior (Bargmann and Horvitz, 1991; Janssen et al., 2000; Wakabayashi et al., 2004, 2005; Zhang et al., 2005; Chang et al., 2006; Hukema et al., 2006; Liang et al., 2006; Dernovici et al., 2007; Zubenco et al., 2008). In each of these ADF-dependent behavior, ADF 5-HT has been shown to be an essential component of the signal; however it is not clear whether increases or decreases in ADF 5-HT are involved. The NSMs are required for food-dependent stimulation of pharyngeal pumping, as well as the enhanced slowing response (ESR) on food, male tail curling and turning during mating (Sawin et al., 2000; Wakabayashi et al., 2004; Avery et al., 1993, Avery and Thomas, 1997; Lipton et al., 2004). However, these studies used ablation of the NSMs as an experimental approach so that it is unclear if NSM 5-HT, glutamate or neuropeptides were involved in the NSM
signal. Whether the ADFs or NSMs sense food associated cues directly or alternatively respond to signals produced from other sensory neurons in response to changes in nutritional state, stress, acute attractive or noxious stimulus is still not clear. Interestingly, we propose 5-HT dependent effects from the NSMs and ADFs are at least due to the synthesized 5-HT from these neurons, due to 1) the overexpression of tph-1 in NSM and ADF both potentiating the serotonin dependent stimulation and inhibition of aversive response to dilute octanol, respectively, and 2) the knockdown of tph-1 in the NSMs abolishing food dependent stimulation of aversive responses.

**INS-1 inhibits NSM and ADF 5-HT release and antagonistically modulates aversive responses**

Neuropeptides are often colocalized with monoamines and in many cases classical neurotransmitters, such as glutamate and acetylcholine (Descaries et al., 2006; Trudeau et al., 2009). However, little is known on the communication between monoamines and peptides released from the same neurons. INS-1 appears to be expressed in the ASI, ASJ, ASH, ADF, ASE, ASG, AWA and BAG sensory neurons, AIA, AIM, RIA and RIC interneurons, NSMs, intestine and vulval muscles, based on fluorescence from an ins-1::venus transcriptional fusion, (Rosoff et al., 1993; Nathoo et al., 2001; Husson et al., 2005, 2006). Three of these neuron types are serotonergic, the NSMs, ADFs and the RIH (Sze et al., 2000, 2002). ins-1 regulates multiple behaviors including, lifespan, food associated preferences involved in thermotaxis, sex specific learning, gustatory and benzaldehyde responses, aswell as chemotaxis, dauer exit and dauer formation/entry under harsh environmental conditions (Kodama et al., 2006; Vellai et al., 2006; Mori et
al., 2005; Chalasani et al., 2010; Lin et al., 2010). More importantly, \textit{ins-1} modulates multiple behaviors that depend on nutritional state either singularly or in combination with other insulin-like peptides. For example, \textit{ins-1}, \textit{ins-9}, and \textit{daf-28} are expressed in ASI and ASJ chemosensory neurons that are critical for dauer formation (Bargmann and Horvitz, 1991). It appears that \textit{ins-1} translates the absence of food into starvation signals that subsequently modify behavior. For example, \textit{ins-1} acts through the AWC sensory neurons to regulate starvation-dependent olfactory responses to odors such as benzaldehyde, and through the ASEs to regulate starvation-induced avoidance responses to salt (Tomioka et al., 2006; Chalasani et al., 2010; Lin et al., 2010). In the present study, INS-1 would appear to inhibit the release of 5-HT from both the NSMs and ADFs, in agreement with the observation that overall 5-HT levels are reduced during starvation. However, since NSM and ADF can have dramatically different roles in behavioral modulation, it will be important to assess the roles of 5-HT release from individual serotonergic neurons during starvation. For example, are NSM and ADF 5-HT release differentially regulated during starvation?

**NSM specific expression of EAT-4 is essential for food dependent stimulation of aversive response to dilute octanol.**

The NSMs are serotonergic, based on the expression of components of serotonergic signaling, including, \textit{tph-1}, \textit{mod-5}, \textit{bas-1} and \textit{cat-1} and their role in the modulation of most key behaviors are ascribed to serotonin release, often without direct evidence of 5-HT involvement (Duerr et al., 1999; Sze et al., 2000, 2002). The NSMs also express \textit{eat-4}, that encodes an ortholog of the mammalian BNPI vesicular glutamate
transporter. *eat-4* is also expressed in a number of other neurons, including, ADA, ALM, ASH, ASK, AUA, and AVJ or AIN, AVM, FLP, IL1, LUA, OLL, OLQ, PLM, PVD, and PVR (Lee et al., 1999; Hiliard et al., 2005; Wakabayashi et al., 2008) and has been previously implicated in most glutamate-dependent behaviors, including, male mating, foraging, reversal frequency (ARS), reversal duration, and ASH-mediated aversive responses to octanol, quinine, SDS, glycerol, copper, high osmolarity, nose touch, as well as AWC-mediated chemotaxis involving isoamyl alcohol, benzaldehyde, pyrazine (Hart et al., 1995; Mellem et al., 2002; Hilliard et al., 2004; Hill et al., 2006; Rose et al., 2004; Chalassani et al., 2007; Harris et al., 2010). The NSMs do not appear to have any obvious synaptic connections, suggesting that if they release glutamate sequestered by EAT-4 it will be released humorally to modulate the downstream ASH-mediated locomotory circuit, possibly via one of the many glutamate receptor subunits that have previously implicated in aversive responses on food (Hart et al., 1995; Kass et al., 2000; Mellem et al., 2002; Hill et al., 2006). Alternatively, NSM EAT-4 may modulate the release of neuromodulators from the NSM directly, either by binding to NSM expressed glutamate-gated channels or metabotropic GPCRs or by modulating the vesicular loading of NSM-expressed neuromodulators, including 5-HT or a potentially wide array of peptides encoded by putative peptide encoding genes that appear to be expressed in the NSMs. For example, the NSMs express *flp-4, nlp-3, nlp-13, nlp-18, nlp-19,* and *ins-1* (Nathoo et al., 2001). Importantly, in mammals, serotonergic neurotransmission is influenced by glutamate vesicular transporters, VGLUTs. In contrast to VGLUT1 and 2 that appear to be expressed in glutamatergic neurons, VGLUT3 is expressed in neurons that are usually classified as non-glutamatergic, suggesting a novel mode of action for
VGLUT3 (Takamori et al., 2002; Juge et al., 2006). VGLUT3 mRNA localizes to limited subsets of serotonergic terminals, as well as cholinergic striatal neurons (Gras et al., 2008; Jackson et al., 2009). In both sets of neurons, VGLUT3 increases both neurotransmission by directly increasing 5-HT and acetylcholine loading into endocytic vesicles (Gras et al., 2008; Amilhon et al., 2010). The mechanism and specificity of VGLUT modulation is unclear, but may involve the maintenance of vesicular membrane potential and/or internal pH. Clearly, NSM EAT-4 is essential for food-dependent stimulation and NSM eat-4 overexpression dramatically enhances food-stimulation. Given the clear similarities with mammalian serotonergic signaling, *C. elegans* may be an especially useful model to dissect the specifics of serotonergic/glutamatergic cotransmission.

**NSM-dependent stimulation and ADF-dependent inhibition of aversive responses requires distinct 5-HT receptors.**

Many studies have identified roles for individual 5-HT receptors in the modulation of key behaviors in *C. elegans*. For example, SER-1 is essential for the 5-HT dependent stimulation of egg-laying, food enhanced slowing response, lifespan, male mating, pharyngeal pumping and reversal frequency on food (Hobson et al., 2003; Carnell et al., 2005; Dempsey et al., 2005; Dernovici et al., 2007; Petrascheck et al., 2007; Zubenco et al., 2008; Harris et al., 2009). SER-4 has been implicated in food dependent enhanced slowing response), male tail curling, foraging, inhibition of egg-laying and lifespan (Horvitz et al., 1982; Dempsey et al., 2005; Carret-Pierrat et al., 2006; Petrascheck et al., 2007; Hapiak et al., 2009). SER-7 is required for stimulation of egg-
laying and pharyngeal pumping by serotonin and also hypoxia-enhanced sensory perception (for regular pumping in response to bacteria, and probably for 5-HT to activate MC neurons (Hobson et al., 2003). MOD-1 is important in the enhanced slowing response, inhibition of egg-laying, stimulation of reversals on food, and food dependent roaming, as well as aversive learning responses to pathogenic bacteria and chemoaversive responses to benzaldehyde in aged animals (Ranganathan et al., 2004; Carnell et al., 2005; Zhang et al., 2005; Dernovici et al., 2007; Tsui and Vander Kooy, 2008; Arous et al., 2009; Harris et al., 2009). Despite the knowledge of 5-HT receptors required for food-associated behaviors, the distinct source of the 5-HT required for each behavior has only been partially characterized. For example, NSM 5-HT appears essential for the enhanced slowing response on food and dispersal behaviors (Sawin et al., 2000; Wakabayashi et al., 2004). ADF 5-HT appears to be the source of serotonergic signaling required for overall food/5-HT dependent effects on hyperoxia, stress responses, foraging, enhancing avoidance responses to pathogenic bacteria and dispersal behavior (Sawin et al., 2000; Wakabayashi et al., 2004; Zhang et al., 2005; Chang et al., 2006; Liang et al., 2006; Zubenco et al., 2008). The HSNs are required for food stimulated egg-laying through direct excitation of the vulval muscles and VC motor neuron, whether 5-HT from the HSNs is responsible for this stimulation is still not clear, as well as modulation of dispersal behavior off food (Weinshenker et al., 1995; Wakabayashi et al., 2004; Zhang et al., 2008). Whether the 5-HT originating from these sources modulates the distinct behaviors synaptically or extrasynaptically or both is still not clear.

Our data suggests that increased NSM 5-HT signaling required for aversive responses requires SER-5 and SER-1, but does not require MOD-1, despite the
observation that MOD-1 is required for the food or 5-HT sensitization of ASH mediated aversive responses. One obvious explanation for this observation is that additional sources of serotonergic signaling contribute to food-dependent increases in aversive responses. For example, synaptic connections between the serotonergic HSNs and the MOD-1 expressing neurons, AIZ, AIA, AIB and AIY, have been described. In addition the HSNs also synapse on the ASHs (White et al., 1986; Ranganathan et al., 2000; Sze et al., 2000).

In contrast, ADF dependent inhibition of aversive responses on food only requires SER-1. This suggests that SER-1 is essential for both ADF inhibition and NSM 5-HT dependent stimulation of ASH-mediated aversive responses. We previously identified a role for the RIA interneurons in SER-1 dependent sensitization; whether SER-1 functions in the RIAs in ADF inhibition remains to be determined (Dernovici et al., 2007; Harris et al., 2009). However, preliminary results involving *ser-1* RNAi knockdown in the RIAs suggests that the RIAs are not involved (Harris, unpublished). These studies are continuing to localize SER-1 in the mediation of ADF 5-HT inhibition.

**Food and 5-HT modulates post-initiation responses through NSM 5-HT and multiple 5-HT receptors**

The present study has demonstrated that food and 5-HT modulate multiple components of the chemoaversive response upon exposure to octanol. For example, in addition to decreasing the time taken to initiate reversal food modulate post-initiation behaviors, including the duration of reversal and directional decisions after termination of reversal. For example, animals on food or 5-HT decrease reversal duration (i.e short
reversals) and return in their initial trajectory after termination of reversal. This observation suggests that nutritional status not only dictates acute responsiveness to stimulus, but also the behaviors performed after the acute response is generated. The idea that food availability modulates reversal duration or frequency has been previously documented in a number of studies (Tsalik and Hobert, 2003; Gray et al., 2004; Wakabayashi et al., 2005; Dernovici et al., 2007; Harris et al., 2009). Interestingly, the presence of food modulates the post-initiation response to the same extent regardless of the intensity of the stimulus (30% vs 100%). Sensory neurons including, AWCs, ASKs, and ASIs have been previously implicated in translating food availability into changes in locomotory dynamics and therefore may be required for modulating the ASH-mediated circuit. For example, AWCs modulate turning and reversals in response to changes in food availability (Chalassani et al., 2007), the ASKs, ASIs and ADFs reversal frequency and reversal duration off food (Wakabayashi et al., 2004; 2005; Chalasani et al., 2007; Wakabayashi et al., 2008; Chalassani et al., 2010). These observations suggest that multiple sensory neurons may control nutritional state dependent locomotory dynamics.
6.0 CHAPTER VI

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